Food Research

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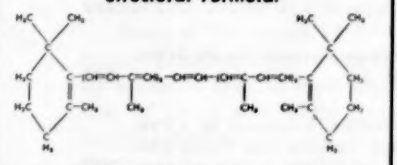

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INFLUENCE OF pH AND TOTAL ACIDITY ON CALCIUM TOLERANCE OF SHERRY WINE

RAUL DE SOTO
Roma Wine Company

AND

HANS WARKENTIN
The Twining Laboratories

(Manuscript received June 28, 1954)

Technological advances in the wine industry are usually accompanied by new problems of stability. By means of its many processing steps the winery is always trying to anticipate an unknown number of expected and unexpected chemical reactions that could make a wine unstable.

Some of the causes of instability have been studied over many generations. Thus, oxidative browning of wines and the undesirable taste caused by excess of iron in wine are mentioned as early as 1874 *(8)*. The formation of black and white iron casse, as well as cream of tartar, calcium tartrate precipitates, and other causes of instability have been studied and discussed by many workers *(1, 2, 3, 5, 6, 7, 9, 12)*.

Most of the information on calcium contents of wines dates back to the end of the 19th century. Von der Heide reviewed this early information in his classical wine treatise, *Der Wein*, and reported that European wines contained from 70 to 140 p.p.m. of calcium with only a few wines as low as 20 or as high as 350 p.p.m. He concluded from his investigations that, due to the insolubility of calcium tartrate, large amounts of calcium cannot stay in solution in wine in the presence of significant amounts of tartaric acid.

Although calcium tartrate and its behaviour were known in Europe for many years, its presence was considered natural and not a stability problem. In California the presence of calcium in wine became a problem gradually. An occasional attempt to reduce wine acidity with calcium carbonate resulted in a wine which would throw a calcium tartrate precipitate from one to six months after bottling. The use of skim milk as a fining agent also resulted in excessive calcium pickup and instability. In the literature in 1935, 1940, and 1941 it was pointed out that calcium pickup from new concrete tanks can be considerable and that the contamination of iron and calcium from poorly manufactured filter pads and filter aid may cause cloudiness *(1, 2, 13)*. It was not until 1951, however, that the subject was again discussed as an adverse economic factor in the production of wine by Crawford *(5)* and De Soto *(7)*. Schreffler and Witzke *(10)* in 1952 also made reference to this calcium problem. This newly aroused interest is highly suggestive of the new problems brought about by excessive calcium pickup from the high calcium processing materials that were being sold to the industry. In 1946 asbestos containing as high as 7.28% of soluble calcium, filter aids containing up to .38%, and carbon with .41% of soluble calcium resulted in large scale calcium stability problems.

The insidious nature of the problem was that wines, apparently stable, would deposit calcium tartrates one to six months after bottling. Attempts to correct this new serious problem were not successful for several years after its beginning in 1946. Therefore, a search was made to obtain lowest possible calcium processing materials and strict specifications for maximum permissible limits were adopted by the industry.

For a time, it seemed that calcium precipitation could be controlled by keeping the calcium content in wine below 100 mg./l. This was accomplished by strict control of processing materials and blending when necessary. However, in 1949 and 1950 it was found that sherries which had an original calcium level of 83 to 95 mg./l. became unstable and precipitation of calcium tartrate proceeded slowly in these wines until a condition of equilibrium was reached between 50-60 mg. calcium per liter.

Crawford reported in 1951 *(5)* that experimental attempts to remove excess calcium from wine by addition of potassium racemate were very discouraging. Attempts made by the industry to reduce calcium content by cooling and/or heating also failed.

In view of the fact that the amount of calcium remaining in solution in sherries after normal precipitation has taken place ranges between 34 and 132 mg./l. (Fig. 1), it became necessary to investigate possible controllable factors which might influence its ability to remain in solution.

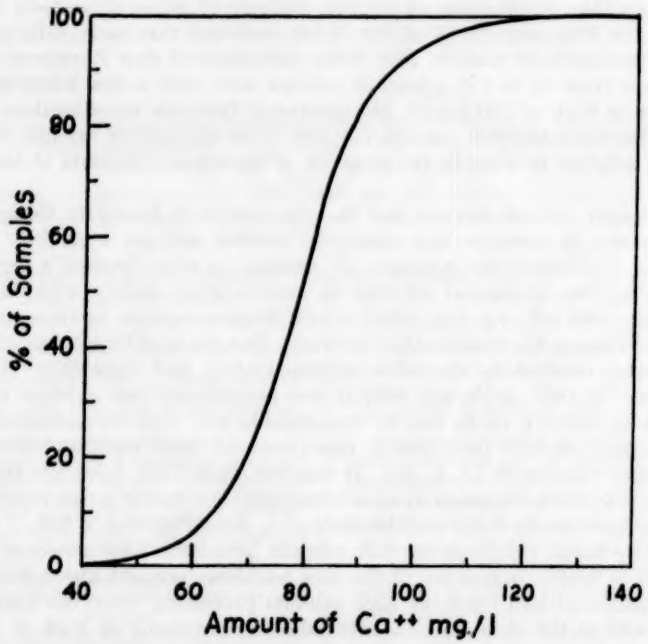

Figure 1. Cumulative frequency distribution of Ca^{++} in sherries. Data based on 388 sherries analyzed over a period of three years.

To find these factors, we must consider the complex nature of wine. Wine is a solution of alcohols, water esters, sugars, tannins, acids and a number of other organic and inorganic compounds. At equilibrium (although this condition may never be fully reached) and at any given conditions of temperature, the relative amounts of each of these components will determine the wine's tolerance for calcium, as well as the amount and rate of formation of calcium tartrate precipitate.

Seidell, in *Solubilities of Inorganic and Metal-Organic Compounds (15)*, reports data by a number of authors on calcium tartrate solubilities in various media. These reported data are quite conflicting but indicate that alcohol depresses calcium tartrate solubility while acetic and tartaric acids, ammonium, potassium and sodium chlorides considerably increase the solubility. There is also considerable increase in solubility with increase in temperature. The data of Marsh *(14)* show a rapid increase in solubility of calcium tartrate in water with decrease in pH.

In sherry wine the amount of alcohol can be varied only within very narrow limits; esters, sugars, and tannins are not subject to drastic change without change in the characteristics of the wine. Nor are the minor constituents adjustable beyond the chilling employed to remove excess potassium acid tartrates in wine. Extended chilling such as is practiced by the wine industry should theoretically also remove excess calcium tartrate but, due to the slow rate of formation of insoluble calcium tartrate, this is not actually the case. This fact left, as the most promising approach, the study of the effects of acidity, since acidity may be changed in a wine within limits without detracting from its quality.

Inspection of data on various plant blends (Table 1) showed a certain correlation between total acidity, pH, and the amount of calcium that apparently could remain in solution in sherry. Preliminary work along these lines indicated that a higher total acidity aided calcium tolerance and plant operation was, therefore, adjusted to higher acidity sherries. This method of control proved to be only partially successful since there were still a few lots that became unstable. Additional observations indicated that pH was a more dependable factor, and an experiment was undertaken to establish the equilibrium conditions of calcium in sherry over a practical pH range.

EXPERIMENTAL

Finished standard sherry with a calcium content of 80 mg./l. and a pH of 3.70 was selected for the experiment.

Because a great deal of the tartrate content originally present in wine is normally lost during processing and stabilization, it was considered desirable to add an excess of tartrate ion to the wine in order to make up for this "deficiency" and, therefore, assure the maximum amount of precipitation possible under experimental conditions. Sufficient tartrate ion (in the form of potassium-sodium tartrate) to combine with 250 mg. of calcium per liter was added to the wine.

In addition to the excess tartrate ion, a small amount (1 ml./l.) of paper-lint suspended in distilled water was also added in order to provide initial nuclei for crystal formation and thus possibly accelerate attainment of equilibrium.

The wine was divided into 5 aliquots and the pH of the aliquots was adjusted to 3.40, 3.60, 3.80, 4.00, and 4.20 by addition of 1 N sodium hydroxide or 1 N hydrochloric acid as required.

TABLE 1

Calcium stability of sherry in plant operation before pH adjustment

Sample	Calcium Mg/1000 cc	pH	Total acid as Tartaric g/100 ml.	$CaC_4H_4O_6$ Precipitate
1	80	3.70	.42	No
2	100	3.60	.40	No
3	117	3.50	.47	No
4	117	3.80	....	Yes
5	82	3.80	....	Yes
6	83	3.70	.38	No
7	73	3.80	.33	Yes
8	85	3.85	.33	Yes
9	78	3.80	.32	Yes
10 (9-B)	73	3.85	.30	No[1]
11	86	3.85	.30	Yes
12	73	3.70	.36	No
13	57	3.75	.39	No
14	54	3.80	.36	No
15	63	3.80	.40	No
16	54	3.80	.40	No
17	59	3.80	.38	No
18	53	3.75	.41	No
19	73	3.75	.42	No
20	93	3.85	....	Yes[2]
21	74	3.82	....	Yes[2]

[1] Same as No. 9—after refrigeration.
[2] Original calcium content calculated from precipitate plus Ca^{++} remaining in solution.

Each of the aliquots was then distributed into 8 bottles. One of the samples out of each pH group was left as a control (80 mg. Ca/l) while the other samples in each group were adjusted to 100, 120, 140, 160, 180, 200, and 220 mg. of calcium per liter by the addition of a neutralized solution of calcium carbonate. The pH was checked on all samples at this point and was found unchanged. Therefore we had 5 pH groups with 8 samples in each group, or a total of 40 samples.

The samples were set aside at normal room temperature (21-22°C.) and observed daily for appearance of precipitate. After a period of 90 days, calcium was determined in the clear supernatant liquid of each sample by the Modified Schwartzenbach Method described by Schreffler and Witzke *(10)*.

RESULTS AND DISCUSSION

A pH range from 3.40 to 4.20 was selected for the experiment because many California wines exist naturally at these pH values and, therefore, the results would supply information of a more practical nature.

The experimental results in Table 2 show the effect of pH on the solubility of calcium tartrate in sherry. Data shown indicate that a definite relation exists between the calcium tolerance and the pH of sherries. Thus, the amount of calcium ion remaining in solution per liter of wine averaged 41 mg. at pH 4.20 while at pH 3.40 it averaged 128 mg. Plotting of the average amounts of calcium remaining in solution against the various pH values, produced a curve (Fig. 2) which shows the equilibrium conditions of the system $Ca^{++} + C_4H_4O_6^{=} \leftrightarrows CaC_4H_4O_6$ in sherry through all pH values from 3.40 to 4.20.

When the data on calcium tartrate solubility in sherry at various pH values (calculated from Table 3) are plotted on semi-log paper with Marsh's

TABLE 2

Effect of pH on the solubility of calcium tartrate in sherry

Original Ca^{++} Content mg./l.	Ca^{++} remaining in Sherry after 90 day precipitation period, at					First visible precipitate in days after start of experiment, at				
	pH 3.4	pH 3.6	pH 3.8	pH 4.0	pH 4.2	pH 3.4	pH 3.6	pH 3.8	pH 4.0	pH 4.2
80	81 [1]	79 [1]	60	51	41	10	8	7	6	6
100	97 [1]	98	64	52	42	10	8	7	6	5
120	116 [1]	98	64	50	40	9	7	6	5	5
140	128	101	64	50	39	8	7	6	5	4
160	130	97	62	51	38	7	6	5	4	3
180	127	98	62	52	44	6	5	4	3	2
200	126	98	60	50	44	4	4	3	2	1
220	129	99	64	52	43	4	3	2	1	1
	128 Av.	98 Av.	63 Av.	51 Av.	41 Av.					

[1] Apparently less calcium is present than sherry can keep in solution at this pH.

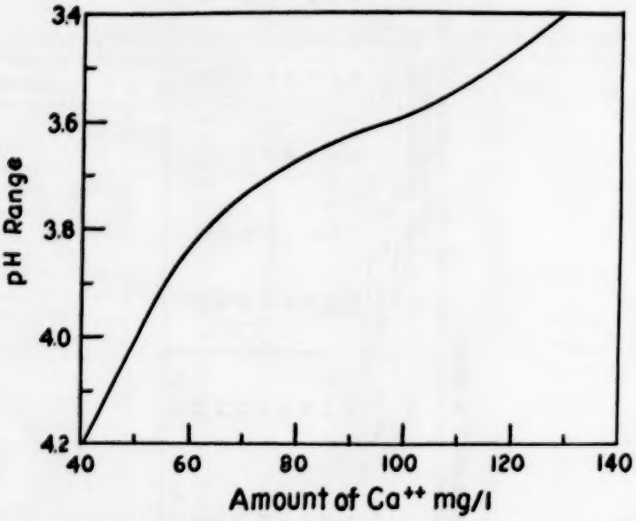

Figure 2. Equilibrium conditions for the system $Ca^{++} + C_4H_4O_6^{=} \leftrightarrows Ca\ C_4H_4O_6$ In sherry at various pH values.

(14) data on solubility of calcium tartrate in water at several pH values, essentially parallel lines are obtained (Fig. 3). This suggests that the rate of solubility increase with decrease in pH is the same in sherry as in water.

The rather slow formation of calcium tartrate precipitate in sherry (Table 2) may partly explain the conflicting solubility values reported in the literature by different authors. It is also unlikely that distilled water of the same pH and calcium tartrate of the same purity were used by all workers. Since a relatively small change in pH produces a rather large change in solubility, such unmentioned or overlooked details may have contributed to the differences in the reported data.

Inspection of Figure 1 shows that 94% of all sherries contained 100 mg./l. of calcium or less while Figure 2 indicates that a pH of 3.59 would be necessary to prevent calcium tartrate precipitation when the amount of calcium ion present is 100 mg./l. The significance of these relations is quite evident, and we should expect, accordingly, 94% of all sherries to be calcium-stable at a pH value of 3.59 or less. The remaining 6% of the sherries which would require a lower pH to keep a larger amount of calcium in solution would be subject to serious calcium tartrate precipitation.

The reason for the failures of the sherries in Table 1 is evident when interpreted in the light of information contained in Figure 2, and a practical approach to the solution of the problem offers itself in the adjustment of the pH of the wine to the level indicated in Figure 2 for any particular calcium content. In some cases, blending with lots containing less calcium would be necessary before a practicable adjustment of pH would be possible.

TABLE 3

Calcium stability of sherry in plant operation after pH adjustment

Sample	Calcium mg/liter	pH	Total acid as tartaric acid, g/100 ml.	$CaC_4H_4O_6$ precipitate
1	72	3.55	.46	No
2	80	3.58	.44	No
3	82	3.70	.47	No
4	82	3.50	.54	No
5	96	3.60	.53	No
6	90	3.50	.44	No
7	96	3.61	.48	No
8	71	3.68	.48	No
9	76	3.73	.46	No
10	78	3.70	.47	No
11	72	3.76	.47	No
12	70	3.74	.47	No
13	80	3.50	.44	No
14	82	3.50	.44	No
15	76	3.72	.46	No
16	72	3.59	.44	No
17	89	3.64	.47	No
18	56	3.77	.47	No
19	89	3.57	.48	No
20	84	3.70	.46	No
21	55	3.60	.47	No
22	84	3.62	.51	No
23	55	3.60	.47	No
24	78	3.64	.40	No
25	80	3.70	.53	No

This method of control has been adapted as standard procedure for plant production of sherries and the results through the last three years have demonstrated a highly gratifying degree of correlation between plant production and laboratory findings. Table 3 shows data of plant production of sherries accumulated during this period. In contrast with Table 1, there are no failures due to calcium tartrate precipitation in these wines.

Experience has shown that in spite of critical selection of processing materials and adoption of all types of safeguards and preventive measures, wines are still subject to contamination with small amounts of calcium. This contamination upsets the equilibrium of the system $Ca^{++} + C_4H_4O_6^{=} \leftrightarrows CaC_4H_4O_6$ and, according to the LeChatelier-Braun principle, manifests itself by precipitation of calcium tartrate. Since no practical means for the removal of this small excess of calcium from wine is known, the only recourse available to the winemaker is the modification of conditions present in the wine so as to increase its calcium tolerance within the possible range of calcium contamination. In experiments performed in the laboratory using approved materials from stock, the calcium content of wine was increased from 78 to 193 mg./l. by using 100 times the amount of diatomaceous filter aid employed in plant operation and from 94 to 136 mg./l. by using 100 times the amount of asbestos pads necessary for plant operation. The reason for such an exaggerated experimental exposure is that in preparing filter pads and aids for a 100,000 gallon filtration, most of the soluble calcium from the processing materials may be dissolved by the first 1,000 gallons of wine.

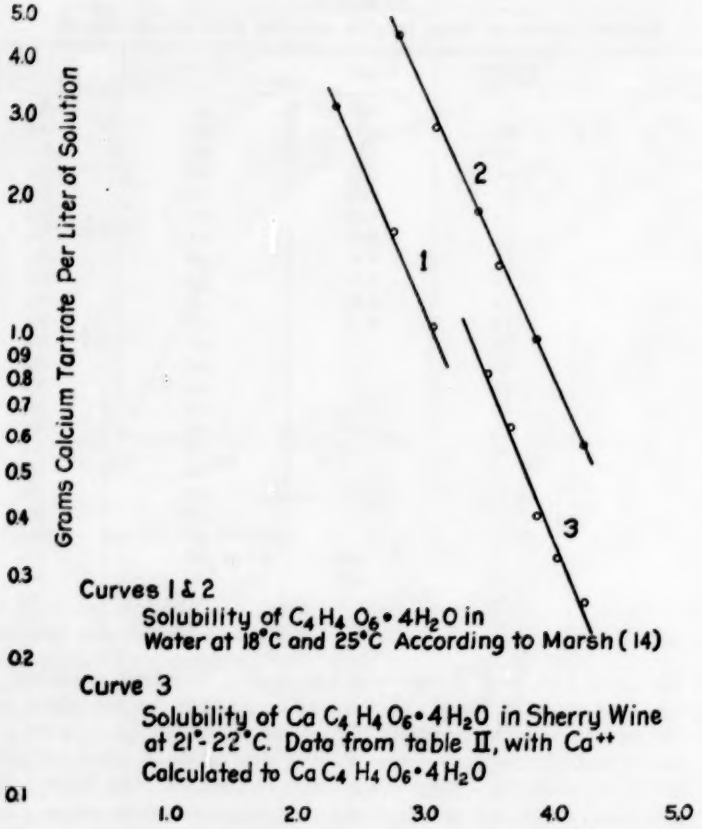

Figure 3. Effects of pH on the solubility of calcium tartrate in water and in sherry wine.

If these 1,000 gallons are bottled before mixing with the other 99,000 gallons, a serious case of calcium instability may occur.

According to Amerine and Joslyn *(1)* pH values of California table wines range from 2.90 to 3.80 while those of dessert wines range from 3.50 to 4.20. Our experimental results explain why table wines with their low pH values were offering no calcium instability problems while dessert wines with their higher pH values produced serious calcium instability.

That pH control can become a valuable production tool is also shown by Costa *(4)* who found that best potassium acid tartrate removal in chill proofing of wine is attained when pH is adjusted to 3.80; by Vaughn *(11)* who recommends a pH not to exceed 3.70 to prevent microbiological spoilage of dessert wines by *Lactobacillus trichoides*, and by Amerine and Joslyn *(1)* who report that white iron casse occurs only in wines with pH values below 3.60. Therefore, while we realize that the above may not be all the problems

that may be solved by a proper and judicious adjustment of pH in wines, the present state of knowledge of this subject seems to indicate that the optimum pH of dessert wines would be somewhere between 3.60 and 3.70.

SUMMARY

Laboratory experiments were made to determine the equilibrium conditions of calcium ion in sherry at various pH values. The knowledge gained from these experiments was successfully applied in plant operations. It was found that when sherries were adjusted to a pH of 3.50 to 3.70, depending on calcium content, calcium instability was prevented in all plant blends.

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INFLUENCE OF MONOSODIUM GLUTAMATE ON TASTE PERCEPTION[a,b]

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The fact that monosodium glutamate (MSG) increases palatability of some foods, as demonstrated by consumer preference studies *(3)*, would place it among the seasonings and condiments. However, statements have been made *(1, 6, 7)* to the effect that MSG is a unique substance that does not impart a flavor of its own but serves only to enhance the natural flavors of foods by increasing the sensitivity of the taste receptors. This might be termed an "accentuation" or "sensitization" hypothesis.

Several investigators have tested this hypothesis. Lockhart and Gainer *(5)* used subliminal concentrations of MSG in supraliminal sugar and salt solutions along with control (no MSG) solutions in triangle tests. The MSG had no significant effect on the taste of sucrose or sodium chloride. In those cases where a change in taste was observed, the taste appeared to be that of glutamate rather than additional sweetness or saltiness. Mosel and Kantrowitz *(8)* determined the effect of exposure to a 5 times threshold concentration of MSG five minutes before measuring absolute thresholds for the four basic tastes. Compared to control thresholds, *sweet* and *salty* were unaffected, acuity to *sour* was increased somewhat and acuity to *bitter* was greatly increased. These authors also discuss other publications, pointing out that there has been confusion of palatability with intensity and that some results are inconsistent with the "sensitization" hypothesis. Another study *(10)* employing subliminal concentrations of MSG (0.75 times threshold) showed a lowering of thresholds for *sweet* and *salt* but not those for *bitter* and *sour*. The results of these three studies do not support the hypothesis that MSG is a general sensitizer for taste acuity. Others who have sponsored this hypothesis do not support it with data.

Our investigation was undertaken to obtain further evidence on the validity of this hypothesis. The experiments included three types of measurements to assess the effects of MSG on gustatory acuity and flavor perception: changes in the absolute threshold (RL); changes in the differential threshold (DL); and, changes in subjective intensity.

PROCEDURE

Materials. The sodium chloride, sucrose and hydrochloric acid were C.P. grade; the caffeine was U.S.P., and the MSG was commercial 99+% pure. Water for preparing

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[a] This paper reports research undertaken at the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 520 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

[b] Based on a paper presented at the 62nd Annual Meeting of the American Psychological Association, New York, N. Y., September 6, 1954.

solutions and for rinsing was distilled and then filtered through charcoal to remove all taste and odor. In all experiments the solutions were presented in 1 oz. glasses; the amount was 6 ml. for the RL's and DL's and about 25 ml. for subjective intensity. The time interval between samples was 30 seconds in all cases.

Subjects. Subjects for the threshold studies all had had considerable experience in laboratory sensory testing of foods and were familiar with the specific methods used. The latter are standard psychophysical techniques *(4)*. For the subjective intensity experiments subjects were drawn from a large group of people normally available for food preference studies.

Subjective intensity. The effect of MSG on weak, but supraliminal, concentrations of the primary taste qualities was determined by the method of paired comparisons. Each pair consisted of identical concentrations of one of the basic taste substances, with one sample of the pair containing MSG in addition to the basic taste substance. The question asked of each subject was: "Which is saltier?" or "Which is sweeter?," etc. The substances and the concentrations employed are shown in Table 1. The results were tested for significance against the expected chance distribution of 50-50.

TABLE 1

Subjective intensity[1] of basic taste solutions with and without added monosodium glutamate (0.2%)

Taste quality	No. of subjects	Substance and conc.		Samples with MSG judged stronger	p[2]
			%	%	
Sweet	35	Sucrose	2.0	34	.09
Sour	48	Hydrochloric acid	0.021	62	.11
Salty	30	Sodium chloride	0.2	80	.002
Bitter	48	Quinine sulfate	.00015	81	.001

[1] Method of paired comparisons. One pair of samples per subject (untrained consumer-observers).
[2] Probability that difference from expected distribution of 50-50 could have occurred by chance.

Absolute threshold. RL's for *sweet* and *sour* were determined by the method of constant stimuli, using 7 concentrations for each subject. The 7 concentrations were chosen from a larger series to encompass the range of each subject's RL. The 7 samples were presented in a different random order to each subject at a session and in a different order for a given subject at successive sessions. Control and experimental sessions were alternated for a total of 10 sessions. For the control sessions the subject rinsed his mouth with water for 5 seconds before each sample; for experimental sessions he rinsed for 5 seconds with 0.068% (0.004M) MSG. He rinsed with water after each sample in both experimental and control sessions. To each sample, the subject responded with a "Yes" or "No" indicating presence or absence of the taste. Another study on the sweet threshold, employing the method of limits, was made to assess the effect of method on the results. Only the ascending order was used, as is common practice in gustatory studies in order to avoid adaptation to the stimulus. In this study each subject rinsed with an MSG solution that was twice the concentration of his own threshold concentration.

Differential threshold. The DL for salt was measured by the method of a single stimuli using concentrations of 0.8, 0.9, 1.0 and 1.1% sodium chloride solutions. At each session each of 18 subjects received the 4 concentrations in ascending order, with knowledge, as a practice set, and then received the series twice in random order to be judged as to strength. The judgments were scored on the basis of proportion of times a sample was judged stronger than the hypothetical mid-point of 0.95% salt. For the control session he rinsed before and after each sample with water; for the experimental session he rinsed before with a 0.17% (.01M) solution of MSG and after with water. For the bitter DL the method of constant stimulus differences was used with .0020M (.039%) caffeine as the standard to be compared to concentrations of .0014, .0016, .0018, .0022, .0024, and .0026M solutions. The order of the pairs, and the position of the standard in the pairs, was randomized. The subject judged which sample in each pair was stronger. Seventeen subjects made a total of 72 judgments per point under both the experimental and control conditions. The rinsing procedures were the same as for salt.

Raw data were converted to proportion judged "Yes" in the case of RL's, and proportion judged stronger than the mid-point in the case of the DL's. Then these proportions were transformed to normal deviates, or sigma values, to convert the psychometric curves to straight lines *(4)*. Lines were fitted to the points by the least squares technique and the resulting equations were then solved for the required 50% and 75% points to obtain the RL's, DL's and PSE's (point of subjective equality).

RESULTS

That MSG has some effect on subjective intensity is evident from Table 1. *Salt* and *bitter* were intensified, but *sour* and *sweet* were not significantly affected. General flavor intensification was not apparent. It is questionable whether even the result for *salt* should be called intensification since a pure MSG solution will be called salty by many people. Hence, the increased saltiness may be only the result of increasing the concentration of salty-tasting solutes. The increase in bitterness cannot be explained as readily in the same way.

If MSG sensitizes the end organs of taste, then thresholds should be lower immediately after the mouth has been exposed to it. Table 2 shows the changes in thresholds for *sweet* and *sour*. Acuity to *sweet* and *sour* is actually decreased. This result for *sour* is the opposite of that obtained by Mosel and Kantrowitz *(8)*. This may be due to methodological differences.

The effect of an MSG rinse on the DL's for salty and bitter qualities is illustrated by data in Table 3. Neither result reaches the usually accepted levels of statistical significance.

DISCUSSION

No consistent pattern of the effect of MSG has emerged from these experiments. General intensification of taste is not evident. Intensity and acuity are reduced in some cases, increased in others, and sometimes there is no effect. These results substantiate those of other investigators. Selective intensification, such as was found in the experiments on subjective intensity, shown in Table 1, is congruent with traditional principles of flavor mixture and requires no special hypothesis.

Other studies from this laboratory *(2)* on preferences have shown that MSG improves hamburger that had developed rancidity due to exposure to ultra-violet light. According to the intensification hypothesis, these off-flavors should have been accentuated with a consequent loss in preference. Furthermore, preference studies have not shown MSG to be consistent in its effects on any one class of foods. For example, it improves some green vegetables and not others, using the criterion of consumer preference.

TABLE 2

Effect of a prior rinse with a monosodium glutamate solution on the RL's for two basic tastes

Taste quality	No. of subjects	Substance and conc.		Conc. of MSG solution	RL[1]	p[2]
			%	%	%	
Sweet[3]	5	Sucrose	0.05-0.7	0	.27	.05
				2 × thresh.	.32	
Sweet[4]	10	Sucrose	.25-.70	0	.34	.01
				.068	.43	
Sour[4]	8	Hydrochloric acid	0.0002-.002	0	.00128	.001
				.068	.00171	

[1] RL = absolute threshold.
[2] Probability that difference could have occurred by chance.
[3] Method of limits.
[4] Method of constant stimuli.

TABLE 3

Effect of a prior rinse with a monosodium glutamate solution on the differential sensitivity for two basic tastes

Taste quality	No. of subjects	Substance and conc. of mid-point	Conc. of MSG	k[1]	p[2]
			%		
Salty	18	Sodium chloride[3] 0.95	0	.119	0.35
			0.17	.123	
Bitter	17	Caffeine[4] 0.039	0	.378	0.12
			0.17	.588	

[1] $k = \frac{DL}{PSE}$ (Weber fraction).
[2] Probability that difference could have occurred by chance.
[3] Method of single stimuli, four points.
[4] Method of constant stimulus differences, six points.

Another line of evidence that has yielded information on the "sensitization" hypothesis can be found in electrophysiological experiments *(9)*. In these experiments the responses of the taste nerves of rats were measured while the tongue was being stimulated with solutions of various salts. It was found that MSG in NaCl solutions did not have an enhancement or persistence effect on the salt response; rather, the effects were merely additive. By itself MSG gave a characteristic salt response at 0.1M.

MSG is a useful seasoning since it does increase preference for a number of foods. In one series of experiments *(3)* about half of a selected group of foods were benefited; improvement was found among foods representing all of the common flavor types other than sweet foods. But since no evidence to date has shown that MSG has any unusual effect on sensory acuity, it may be assumed that the mechanism through which it alters flavors is similar to that of other seasonings.

SUMMARY

Three types of experiments were designed to test the hypothesis that monosodium glutamate (MSG) affects flavor perception by altering gustatory acuity. Absolute and differential thresholds were measured with and without a prior mouth rinse with MSG. The effect of the presence of MSG in solutions of the basic tastes on subjective intensity was determined.

MSG raised the absolute thresholds for *sweet* and *sour;* it did not significantly alter the differential thresholds for *salt* and *bitter.* It increased the subjective intensities of *salt* and *bitter,* but had no effect on sour and sweet tastes.

No consistent pattern of the effect of MSG emerged. The results do not support the hypothesis that MSG acts as a general intensifier of flavor and suggest no other hypothesis, except the general one that MSG is simply another seasoning that may contribute a flavor of its own to a complex food flavor.

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THE INFLUENCE OF CATIONIC ENVIRONMENTS ON THE THERMAL RESISTANCE OF *BACILLUS COAGULANS*[a,b]

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When heated in different foods, an organism may have different degrees of thermal resistance *(2, 25, 26)*. Many factors in foods contribute to these differences. It has been reported that the nature of the suspending medium during heat treatment has a marked effect on resistance *(6, 17, 23, 25, 29, 30)*. Sugiyama *(27)* reported that as the concentration of calcium or magnesium ions was increased in the suspending medium, the thermostability of spores of *Clostridium botulinum* increased. The effect of hydrogen ion concentration on resistance is well known. It is generally agreed that at pH values below 5 and above 10 resistance is decreased *(6, 10, 15, 22)*. Viljoen *(29)* reported that NaCl increased the resistance of various obligate and facultative anaerobic sporeformers, and Anderson, Esselen, and Fellers *(2)* demonstrated this effect of NaCl on the thermal resistance of *Bacillus coagulans*. Esty and Meyer *(6)*, reporting on *Cl. botulinum*, stated that minute concentrations of NaCl enhanced resistance, while greater concentrations decreased resistance appreciably.

Since bacteria possess large surface areas in relation to their volume, and since this surface possesses a negative electrostatic charge, positively-charged ions will be attracted from the environment in accordance with fairly rigid physicochemical laws *(11, 12, 13)*. In view of this fact as well as the knowledge of the effects of various cations on the thermal resistance of some sporeformers, thermal resistance studies were carried out on the spores of *Bacillus coagulans* in controlled cationic environments. A group of cations, H^+, Na^+, K^+, Ca^{++}, Mg^{++}, and Fe^{+++}, was selected from those normally present in commercial evaporated milk and tomato juice—two of the foods commonly affected by "flat sour" spoilage caused by this organism *(3, 9, 20, 31)*. A series of tomato juice cation solutions (TJCS)—at pH 4.4—was made up in which cations were deleted singly, and thermal resistance determinations were made in each of these solutions. By comparing the resistance of *B. coagulans* spores in these deletion menstrua with their resistance in TJCS (pH 4.4), the influence of individual cations on resistance was studied.

EXPERIMENTAL METHODS

Production of spore suspension. Screw-cap bottles containing proteose-peptone acid agar (PPAA) *(24)*, as modified by Anderson, Esselen, and Fellers *(2)*, were inoculated

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with a 48-hour culture of *Bacillus coagulans*.* After incubation for 2 weeks at 131°F. (55°C.), the surface growth was removed with sterile distilled water, homogenized for 5 minutes in a Waring blender, and filtered through a glass wool pad. The suspension was washed 3 times with sterile distilled water by centrifugation, resuspended in sterile distilled water, and stored at 40°F. (4.4°C.) in bottles containing about one-half inch of glass beads.

Estimation of spore concentration. The method of Stumbo, Murphy, and Cochran *(26)*, using the thermoresistometer, was employed in the estimation of spore concentration. The stock suspension was diluted in sterile distilled water to give a final concentration of less than one spore per 0.01 ml. Aliquots of 0.01 ml. of the diluted suspension were measured into 90 sterile thermoresistometer cups. After being subjected to a heat "activation" of 0.14 minutes at 220°F. (104.4°C.) *(32)*, the spores were inoculated into tubes of modified PPAA medium and incubated at 98.6°F. (37°C.). The tubes were examined periodically for growth as evidenced by colony formation. After incubation for 2 weeks, a final estimation of the spore count was made by the method of Stumbo *et al. (26)* with the equation of Halvorson and Ziegler *(8)*.

A modification of the PPAA medium of Anderson *et al. (2)* was used for spore counts and thermal resistance studies. Instead of K_2HPO_4, 5 g. of KH_2PO_4 were added per liter of medium, thereby eliminating the necessity of adjusting the pH of the medium. After sterilization, the pH was approximately 5.4. Comparative spore counts demonstrated that the modified medium was superior to the PPAA medium of Anderson *et al. (2)*.

The average of 4 replicate spore count determinations was 2.35×10^8 per ml.

Preparation of suspending menstrua. Commercial evaporated milk and tomato juice were analyzed for 6 cations by the following method:

The products were evaporated to dryness and digested with concentrated nitric acid. After repeated digestion, concentrated hydrochloric acid was added to convert the cations to their chloride salts. The volume was reconstituted with double-distilled water and the samples analyzed for cations.

Potassium, sodium, and calcium were determined by flame photometry using the Beckman Model B flame spectrophotometer. Magnesium was determined by the colorimetric method of Young and Gill *(33)* and ferric ion by the colorimetric method of Fortune and Mellon *(7)*. The pH of the original samples was determined prior to digestion with the Beckman Model H-2 pH meter.

On the basis of the data obtained and those reported in the literature *(1, 4, 19, 28)*, a solution composed of these cations was prepared for each of the 2 foods studied. Table 1 shows the concentrations employed.

The following suspended media were prepared for use in thermal resistance determinations:

1. Evaporated milk ..... pH 6.2
2. Tomato juice ..... pH 4.4
3. EMCS ..... pH 6.3
4. EMCS ..... pH 4.3
5. TJCS ..... pH 6.2
6. TJCS ..... pH 4.4
7. TJCS minus Na^+ ..... pH 4.5
8. TJCS minus K^+ ..... pH 4.6
9. TJCS minus Ca^{++} ..... pH 5.1
10. TJCS minus Mg^{++} ..... pH 5.2
11. TJCS minus Fe^{+++} ..... pH 4.8

Sterile evaporated milk was transferred aseptically to sterile screw-cap bottles. Tomato juice was filtered through Whatman No. 1 paper to remove pulp, transferred to screw-cap bottles, and autoclaved for 30 minutes at 250°F. (121.1°C.). All cation solutions were sterilized by Seitz-filtration. The pH of several of the TJCS "deletion"

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* *Bacillus thermoacidurans* (Strain 43P) obtained from Mr. C. W. Bohrer, National Canners Association Laboratories, Washington, D. C. For proper classification of this organism, see Smith, Gordon, and Clark *(20)*.

TABLE 1

Composition of tomato juice cation solution (TJCS) and of evaporated milk cation solution (EMCS)

Ion	Concentration of cation in g./liter		Salt used
	TJCS	EMCS	
Na^{+}	2.3000	1.0000	NaCl
K^{+}	2.5000	2.5000	KCl
Ca^{++}	0.0750	0.7222	$CaCl_2$
Mg^{++}	0.0750	0.2000	$MgCl_2 \cdot 6H_2O$
Fe^{+++}	0.0040	0.0015	$FeCl_3 \cdot 6H_2O$

series was altered during filtration in spite of the citric acid-phosphate buffer system *(5)* used in all suspending solutions. The amount of citric acid in the buffer system was below the inhibitory concentration reported for this organism by Murdock *(14)* and below that in tomato juice *(18)*.

Determination of thermal resistance. Thermal resistance values of *B. coagulans* suspended in each of 11 menstrua were determined by the method of Stumbo *et al. (26)* for 2 concentrations of spores subjected to 4 temperatures (220, 225, 230, and 235°F.) (104.4, 107.2, 110, and 112.7°C.). The spore suspension was diluted in sterile distilled water. Ten ml. of the diluted spore suspension were transferred to 90 ml. of the menstruum under study, to give the desired concentration, and placed overnight on a mechanical shaker at 38 to 40°F. (3.3 to 4.4°C.). Samples of 0.01 ml. of the suspending menstrua were transferred aseptically to sterile thermoresistometer cups. For a given concentration, sets of 6 samples each were subjected to a series of 7 different time exposures at a particular temperature, inoculated into modified PPAA, and incubated at 98.6°F. Periodically, the tubes were examined for growth as evidenced by colony formation.

After incubation for 2 weeks, a D value was calculated as follows for each exposure time that sterilized only a fraction of the samples per set *(26)*:

$$D = \frac{U}{\log a - \log b}$$

where

D = time in minutes necessary to accomplish a 90% reduction in the number of spores.
U = heating time in minutes.
a = number of spores subjected to one time-temperature relationship (the number initially present per sample multiplied by the number of replicate samples—$23.5 \times 6 = 141$ and $235 \times 6 = 1410$ in the present investigation).
b = number of spores surviving at the end of heating time U.

The value of *b* was obtained by application of the Halvorson and Ziegler *(8)* equation:

$$\bar{x} = 2.3026 \log \frac{n}{q}$$

where

$\bar{x}$ = most probable number of spores surviving per replicate sample
n = total number of replicate samples
q = number of sterile samples

then

b = $\bar{x}$ times the number of replicate samples (i.e., $b = 6\ \bar{x}$ for the method employed in this study).

The values obtained at each exposure time were then averaged, and this value was taken as one replicate D value for that temperature. Table 2 shows the over-all D values obtained from 3 such replications for each of the 2 concentrations at the 4 temperatures employed.

TABLE 2

Thermal resistance values for spores of Bacillus coagulans in various suspending menstrua

Suspending menstrua	pH	D220		D225		D230		D235	
		Initial spore concentration per six sample lot							
		141	1410	141	1410	141	1410	141	1410
Evaporated milk	6.2	2.35	2.43	0.970	0.990	0.348	0.359	0.1610	0.1890
Tomato juice	4.4	1.18	1.12	0.431	0.474	0.195	0.219	0.0943	0.0977
EMCS	6.3	1.32	1.34	0.477	0.539	0.241	0.227	0.0818	0.0923
EMCS	4.3	0.96	1.00	0.413	0.462	0.185	0.200	0.0596	0.0714
TJCS	6.2	1.96	1.95	0.685	0.718	0.259	0.293	0.1100	0.1180
TJCS	4.4	1.15	1.35	0.475	0.515	0.170	0.235	0.0886	0.0954
TJCS minus Na^{+}	4.5	0.74	0.94	0.317	0.296	0.128	0.169	0.0607	0.0845
TJCS minus K^{+}	4.6	1.01	1.04	0.340	0.466	0.178	0.175	0.0800	0.0928
TJCS minus Ca^{++}	5.1	1.32	1.57	0.532	0.610	0.225	0.227	0.1020	0.1040
TJCS minus Mg^{++}	5.2	1.61	1.71	0.618	0.625	0.245	0.294	0.1040	0.1040
TJCS minus Fe^{+++}	4.8	1.08	1.33	0.496	0.538	0.199	0.250	0.0915	0.1060

RESULTS AND DISCUSSION

The data in Table 2 show the differences in thermal resistance (i.e., D) values of *B. coagulans* spores suspended in various menstrua.

The D values obtained for the 2 initial concentrations of spores (see Table 2) at 4 temperatures in 11 suspending menstrua were subjected to an analysis of variance *(21)* to determine the significance of differences of thermal resistance in various menstrua. A transformation of D [i.e., log (D + 10)] was employed in the analysis.

The following paired comparisons were made of D values obtained under the various conditions of suspending menstrua:

1. Evaporated milk and tomato juice**
2. TJCS (pH 4.4) and tomato juice
3. TJCS (pH 4.4) and TJCS (pH 6.2)**
4. EMCS (pH 6.2) and evaporated milk**
5. EMCS (pH 6.3) and EMCS (pH 4.3)**
6. EMCS (pH 4.3) and TJCS (pH 4.4)**
7. EMCS (pH 6.3) and TJCS (pH 6.2)**
8. TJCS (pH 4.4) and TJCS minus Na^{+} (pH 4.5)**
9. TJCS (pH 4.4) and TJCS minus K^{+} (pH 4.6)**
10. TJCS (pH 4.4) and TJCS minus Ca^{++} (pH 5.1)**
11. TJCS (pH 4.4) and TJCS minus Mg^{++} (pH 5.2)**
12. TJCS (pH 4.4) and TJCS minus Fe^{+++} (pH 4.8)

** Indicates significance at the 1% level.

The thermal resistance of these spores is greater in evaporated milk than in tomato juice. On the basis of the differences in cation composition of these 2 foods, especially the H^{+} concentration, a difference in thermal resistance is not surprising. The effect of variation in suspending media has been reported for other organisms as well as for *B. coagulans (2, 14, 18, 22, 26)*.

When the pH was lowered in each of the 2 cationic solutions, thermal resistance was significantly decreased. While the resistance in TJCS at pH

4.4 was similar to that in tomato juice, the resistance in EMCS at pH 6.2 was significantly lower than in evaporated milk. Evaporated milk, then, contains some factor(s) not present in EMCS which enhances the thermal resistance of *B. coagulans* spores. It is possible that this factor(s) is organic in nature.

A comparison of the 2 cation solutions at the same pH indicated that the environments as a whole affected thermal resistance. The resistance of *B. coagulans* spores was higher in TJCS than in EMCS at both pH levels tested.

To evaluate the effect of individual cations, the series of TJCS solutions from which single cations were deleted was compared with unaltered TJCS at pH 4.4. Different D values in the deletion series from those obtained in TJCS at pH 4.4 could then be attributed to the deleted cation.

The thermal resistance of *B. coagulans* spores suspended in the Na^+-deficient solution was significantly lower than in the unaltered TJCS. Since the concentration of Na^+ in TJCS is twice as great as in EMCS, this may contribute to the greater resistance of *B. coagulans* spores in TJCS. The effect of K^+ on thermal resistance parallels that of Na^+. The deletion of K^+ from TJCS resulted in a significant decrease in thermal resistance. Anderson *et al. (2)* reported that by increasing the concentration of NaCl the thermal resistance of *B. coagulans* was decreased. O'Brien *(16)*, in this laboratory, found that when Na^+ or K^+ was added to pea puree, the resistance of P.A. No. 3679 increased.

From Table 2 it appears that the deletion of either Ca^{++} or Mg^{++} from TJCS increased thermal resistance. However, after correcting for the pH rise in these 2 solutions from 4.4, it was found that the thermal resistance values in the Ca^{++}- and Mg^{++}-deficient solutions were not significantly different from those of the control menstruum (TJCS). It was, therefore, concluded that Ca^{++} and Mg^{++} did not alter the thermal resistance. The deletion of Fe^{+++} did not alter the thermal resistance significantly. It is conceivable that this may be due to its low concentration.

In several instances (TJCS at pH 4.4, TJCS minus Na^+, TJCS minus Ca^{++}, and TJCS minus Fe^{+++}) the concentration of spores gave D values which were significantly different from one another. It is felt that these differences are due to experimental variation rather than to defects in the validity of the logarithmic nature of the order of death of spores of *B. coagulans*. Youland and Stumbo *(32)*, using the thermoresistometer method with the same strain of this organism, found that no significant differences in D values existed for 4 concentrations of spores of *B. coagulans* at 4 temperatures, thus substantiating the logarithmic order of death for spores of this species.

SUMMARY

The influence of various cationic environments during heating on the thermal resistance of *Bacillus coagulans* spores was studied by the thermoresistometer method. Thermal resistance was greater in evaporated milk than in tomato juice. Resistance was lower at the lower pH level (pH 4.3) than at the higher pH level (pH 6.2) in both evaporated milk cationic

solution (EMCS) and in tomato juice cationic solution (TJCS). At pH 4.4 and at 6.3, the thermal resistance was higher in TJCS than in EMCS at comparable pH levels.

The deletion of Na^{+} or of K^{+} from TJCS at pH 4.4 resulted in a statistically significant decrease in the thermal resistance of *B. coagulans* spores. The deletion of Ca^{++}, Mg^{++}, or Fe^{+++} did not cause significant changes in thermal resistance.

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THE RESPIRATORY ENZYMES OF MEAT. II. THE TEMPERATURE STABILITY OF BEEF AND PORK SUCCINOXIDASE

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Succinic dehydrogenase, α-glycerophosphate dehydrogenase, and cytochrome oxidase appear to be the chief extant oxidative pathways of meat *(3)*. Their strong activity, relative to other Krebs cycle systems, warrants a close inspection of their stability. In the present study, the stability of succinoxidase (succinic dehydrogenase plus cytochrome oxidase) is examined under several conditions of cooler storage, alternate freezing and thawing, and heating.

EXPERIMENTAL

The effects of cooler storage and alternate freezing and thawing. Treatments were carried out on both unground and ground beef muscle. The former consisted of strips approximately 2.5 cm. × 2.5 cm. × 10 cm., the latter of strands 0.3 cm. in diameter prepared in a hand grinder. The conditions and results are given in Table 1.

In experiments 71 and 75, the beef samples were packed into beakers, covered with wax paper (punctured for entry of the thermometer), and set alternately in a dry ice-acetone mixture and a constant temperature water bath. Temperature equilibrium was avoided to induce continuous change in number and size of ice crystals. Although the thermal conductivity of ice exceeds that of water, the rates of temperature rise and fall were approximately equal during the short periods used.

The effect of infra red heating. Beef tenderloin strips were exposed to heat from an infra red source until the desired internal temperature was reached. It required 2.4 min. to reach 43.4°C. (110°F.) and approximately 1 min. for each subsequent 5.5°C. rise. Following treatment, the strips were placed in a 4°C. cooler until assay.

The effect of oven heat on pork succinoxidase. Ground pork, prepared from fresh trimmings, was packed into 100 ml. beakers and placed in an oven at the desired temperature. Several samples were pre-mixed with chemicals (Table 2). After heat exposure, the meat was stored at 4°C. until assay.

Measurement of succinoxidase. The enzyme was assayed at 37°C. by the method of Schneider and Potter *(6)*. Duplicate results agreed within 10%.

RESULTS

Beef muscle succinoxidase activity was far better preserved in the frozen state than at 4°C. (Table 1). In the latter case, 89% of the activity in ground beef was lost within 4 days (exp. 32: C/B), and 38% of the activity of unground beef was lost within 5 days (exp. 60: B/C).

After rapid alternate freezing and thawing, ground beef showed a 46% greater activity than the control stored at 4°C. (exp. 75). Similar treatment of unground rib muscle produced an activity decrease of only 23% (exp. 71).

When alternate freezing and thawing of unground muscle was gradual, the incurred activity losses were 22% (exp. 39: A/B) and 36% (exp. 39: D/E).

Pre-Assay exposure to infra red heating produced no measured change in beef succinoxidase below 43.4°C. (110°F.). At 48.9°C. (120°F.) the beginning of a precipitous destruction of activity was noted. This effect was linear, one-third of the initial activity being lost in each of 3 increments of 5.5°C. (10°F.).

TABLE 1

The influence of cooler storage and alternate freezing and thawing on beef succinoxidase

Exp.	Meat	Condition	Exposure time at various temperatures						Q_{O_2}	Relative activity
32			− 20°C.	+ 4						
A	rump	strips	4 days	.....					2.6	100
B	rump	ground	4 days	.....					1.9	73
C	rump	ground	.....	4 days					0.2	8
60			− 20°C.	+ 4	+ 21	+ 4				
A	tenderloin	strips	99 hrs.	.....	1 hr.	21 hrs.			6.8	128
B	tenderloin	strips	.....	121 hrs.	.....	.....			3.3	62
C	tenderloin	strips	120 hrs.	.....	1 hr.	.....			5.3	100
71			− 20°C.	− 20 to + 1	+ 1 to − 20	− 20 to + 1	+ 1 to − 20	− 20 to + 4		
A	rib	strips	.....	10 min.	10 min.	10 min.	10 min.	10 min.	10.4	77
B	rib	strips	50 min.	.....	.....	.....	.....		13.5	100
75			+ 4°C.	+ 4 to − 3	− 3 to + 1	+ 1 to − 3	− 3 to + 1	+ 1 to − 20		
A	rib	ground	.....	3 min.	3 min.	3 min.	3 min.	10 min.	3.5	146
B	rib	ground	22 min.	.....	.....	.....	.....	.....	2.4	100
39			+21°C.	+ 50	− 20	+ 21	− 20			
A	sirloin	strips	5 min.	5 min.	10 days	3 hrs.	7 days		1.8	50
B	sirloin	strips	5 min.	5 min.	10 days	.....	.....		2.3	64
C	sirloin	strips	70 min.	.....	10 days	.....	.....		2.9	80
D	sirloin	strips	5 min.	.....	10 days	3 hrs.	7 days		2.3	64
E	sirloin	strips	5 min.	.....	10 days	.....	.....		3.6	100

TABLE 2

The effect of heat on pork succinoxidase and the influence of "stabilizing" chemicals

Exp. No.	Heat treatment		Additions		Pre-heating storage period Hours	Q_{O_2}	Activity loss %
	Temp. °C.	Time hours	Chemical	Conc. %			
109	....	....	.....	....	....	7.8	....
109	60	2	.....	....	....	3.2	59
109	60	6	.....	....	....	1.2	85
111	....	....	.....	....	....	7.3	....
111	60	15.5	.....	....	....	0.8	89
111	60	15.5	calcium phosphate gel	9	0	0.2	97
111	60	15.5	gelatin	3	0	0.7	90
113	....	....	.....	....	....	12.6	....
113	55	1	.....	....	....	7.8	38
113	55	1	sodium succinate	3	2	5.0	60
113	55	1	glucose	3	2	6.8	46
113	55	1	gelatin	3	2	7.3	42

Table 2 illustrates the lability of pork muscle succinoxidase to heat. It further shows the failure of several classes of additives to prevent the enzymatic destruction. Succinate, the principal substrate, unexpectedly appeared to bolster this destruction. This may be an ion effect.

DISCUSSION

There has been no general agreement about the influence of freezing and thawing on respiration in excised tissue. Smith *(8)* reported large losses in resting oxygen consumption of frozen and thawed frog muscles. Brooks *(1)*, working with ox muscle, found no meaningful effect. Woodroof and Shelor *(9)* cited substantial rise in oxidative enzyme activity of frozen and thawed fruit. Apparently, the response to freezing and thawing of the oxidative apparatus in a given excised tissue is not easily predictable. To the many variables governing the course of denaturation in a purified, rigidly defined protein system *(5)*, there are undoubtedly added complex protein-protein interactions and ill-defined salt effects. This is evidenced in the present study by the increase and decrease, respectively, of succinoxidase in ground and intact beef muscle which had been frozen and thawed.

Keilen and Hartree *(4)* and Slater *(7)* have underscored the importance to stability of mutual physical accessibility of succinoxidase components. During 30 days of storage at + 4°C., succinic dehydrogenase activity in heart muscle was far more stable than succinoxidase activity. This result was ascribed to disruption of the colloidal framework binding the catalysts. This type of enzymatic loss, produced also by freezing and drying, was partially reversed by the incorporation of gels and denatured proteins capable of giving new surfaces.

In an effort to exploit these findings, calcium phosphate gel and gelatin were mixed with pork muscle before heat treatment. They gave no protection to the enzyme system. Glucose, which has been reported to augment protein thermal stability *(2)*, was also ineffective.

SUMMARY

Muscle succinoxidase was examined under the influence of cooler storage, alternate freezing and thawing, and heating. Storage in the frozen state

preserved the activity better than storage at +4°C., and at +4°C. the enzyme system in unground muscle was less unstable than that in ground muscle. Losses incurred as a result of freezing and thawing were mild, the highest being 36%. No notable differences emerged to distinguish the effects of rapid and slow alternate freezing and thawing.

Succinoxidase in excised muscle is heat labile; discernible destruction began at 43°C. Colloids, succinate, and glucose failed to protect against heat destruction.

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THE IDENTIFICATION OF SOME VOLATILE CONSTITUENTS OF CONCORD GRAPE JUICE[a]

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In the classical procedure for the identification of the volatile constituents of fruits, a volatile oil is prepared from 100 to 1000 kg. or more of fruit. The volatile oil is fractionated by distillation or by chemical or chromatographic procedures, and the fractions are identified *(2, 3, 4, 5, 7, 8, 15, 16, 20)*. In the present article, a procedure is described which should prove useful for the identification of volatile constituents of fruits starting with as little as 10 kg. of material. In this new procedure a volatile oil is not isolated, but instead the volatile constituents are identified in aqueous solution, usually by preparation of water-insoluble derivatives which are identified chromatographically.

Two other features of this new procedure are noteworthy. A total carbon determination has been introduced in order to obtain a material balance and thus minimize the possibility of overlooking a major volatile constituent. Techniques have been devised for the identification of esters in an aqueous solution containing a large amount of free alcohols. Ordinarily in the identification of an ester, the ester is saponified and the acid and alcohol components are identified. This general approach is satisfactory for the identification of the acid components of esters in the presence of alcohols. However, free alcohols must be removed before saponification of esters if the alcohols formed by the saponification are to be detected. This has been accomplished by using platinum black-catalyzed oxidation to remove the free alcohols *(14)*. Once the free alcohols are removed, the esters are saponified and the alcohol components of the esters are determined in the aqueous solution.

The procedure is described below as it has been used in the identification of the volatile constituents of Concord grape juice (*Vitis labruscana* Bailey). Less than 10 liters of grape juice sufficed for the complete analysis, with all determinations run in duplicate.

EXPERIMENTAL

High quality commercial (1953) Concord grape juice produced by pressing after the grapes were heated to 150°F. (66°C.) was subjected to flash vacuum distillation followed by fractionation of the distillate *in vacuo* (15 in. vacuum), in an apparatus similar to that described by Milleville and Eskew *(12)*. The essence,[b] 1.2% of the original volume of the juice, had an odor very close to, but stronger than, that of the original juice. The essence was subjected to the various analytical procedures described below. It is anticipated that the procedure would be the same with other fruits, after

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[b] We are greatly indebted to the Welch Grape Juice Company, Westfield, N. Y. for the gift of this essence.

preparation of a similar essence, using techniques designed to recover the natural aroma of the fruit.

Identification of alcohols. The alcohols in the essence were identified as 3,5-dinitrobenzoates, according to the method for the identification of alcohols in dilute aqueous solution *(6)*. Because of the high concentration of alcohols in the essence, 1.0 ml. of essence was used in each analysis. The results were made quantitative by comparison with the results obtained from solutions of known mixtures of ethanol and methanol in water.

Identification of esters. *Removal of Free Alcohols.* In a 3-liter glass-stoppered flask was placed 20 ml. of essence and 200 mg. of platinum black *(21)*, and the mixture was shaken mechanically at room temperature for 72 hours. (The large flask was required in order to furnish sufficient oxygen in the air space to complete the oxidation of the alcohols. The flask must be stoppered to prevent losses of esters to the atmosphere during the long oxidation process.) An additional 50 mg. of catalyst was added after 2 hours, and 75 mg. after 24 hours. Aliquots (1 ml.) were removed at 24, 48, and 72 hours, and were titrated with 0.1 *N* sodium hydroxide. (Primary alcohols are oxidized to acids.) The titration at 72 hours was the same as that at 48 hours, indicating that the oxidation was complete in 48 hours.

Identification of Alcohol Components of Esters. The catalyst was removed by filtration, and half of the solution was treated with 3,5-dinitrobenzoyl chloride according to the usual procedure for the determination of alcohols in aqueous solution *(6)*. Chromatography of the 3,5-dinitrobenzoates showed that only traces of ethanol and methanol remained in the solution after oxidation. The other half of the solution was mixed with 1.4 ml. of 5 *N* sodium hydroxide and was heated in a stoppered flask (stoppered to avoid loss of alcohols during the heating period) for 2 hours in a boiling water bath. Excess sodium hydroxide was neutralized with 1 *N* hydrochloric acid, and the solution was treated with 3,5-dinitrobenzoyl chloride. Chromatography of the 3,5-dinitrobenzoates showed that after saponification the solution contained considerable ethanol and some methanol, in the ratio of approximately 20:1. (Controls indicated that during the oxidation procedure there was not a significant amount of esterification or transesterification.)

Identification of Acid Components of Esters. A mixture of 5 ml. of essence and 8.0 ml. of 0.120 *N* sodium hydroxide was refluxed 2 hours. The solution was titrated, to a weak phenolphthalein end-point, with 0.75 ml. of 1.03 *N* hydrochloric acid. The solution was evaporated *in vacuo* to approximately 1 ml., acidified with 0.05 ml. of 1:1 sulfuric acid:water and diluted with water to 2.5 ml. A mixture of 1.7 ml. of this solution, 3.0 g. of silicic acid *(22)*, and 20 ml. of chloroform was made into a slurry, placed at the top of a silicic acid column and the material was chromatographed *(11, 17)*. The only acid found was acetic acid, and within experimental error the amount of acetic acid found accounted for all the ester in the essence, as determined by the saponification equivalent.

Since acetic acid was the only acid component of the esters in the essence, the ethanol and methanol, found as the alcohol components of the esters, must have been present as ethyl acetate and methyl acetate respectively.

Identification of carbonyl compounds. The carbonyl compounds were precipitated from the essence as 2,4-dinitrophenylhydrazones, and the 2,4-dinitrophenylhydrazones were determined chromatographically by the method of White *(19)*. In each experiment, 5.0 ml. of essence was mixed with 5 ml. of an 0.4% solution of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. The solution was allowed to stand overnight in the refrigerator. The mixed 2,4-dinitrophenylhydrazones, weight 5 mg., were chromatographed on bentonite-celite *(19)*. For comparison, mixtures of acetaldehyde and acetone 2,4-dinitrophenylhydrazones were also chromatographed.

Methyl anthranilate. Methyl anthranilate was determined by the standard method *(1)*. Its concentration in the essence was too low for the anthranilic acid to be detected as an acid component of the esters. [To establish the identification of the compound, a derivative, 1-(2′-carbomethoxyphenylazo)-2-naphthol, was prepared from methyl anthranilate isolated from Concord grape juice, and was found to have the same melting point and mixed melting point as that of authentic derivative prepared from methyl anthranilate.]

Identification of acids. The essence did not contain a significant amount of acid. However, the original flash distillate (not fractionated) did contain acid. This acid was isolated as the sodium salt and chromatographed on silicic acid *(11, 17, 22)*. The only acid found was acetic acid. Its absence from the essence is understandable since acetic acid does not form an azeotropic mixture with water and would have been removed from the essence as high-boiling material during fractional distillation.

Analysis for total carbon. Aliquots of approximately 0.10 and 0.20 ml. of essence were subjected to the Van Slyke-Folch *(18)* wet combustion procedure. The carbon dioxide was absorbed in dilute sodium hydroxide in a special absorption tube, and was determined gravimetrically as barium carbonate.

Specific gravity. The specific gravity of the essence was 0.9929 at 20°C. (68°F.). This is the specific gravity of an aqueous ethanol solution containing 39 mg./ml.

Ultraviolet absorption spectrum. The ultraviolet absorption spectrum of the essence is shown in Figure 1 (1 cm. cell).

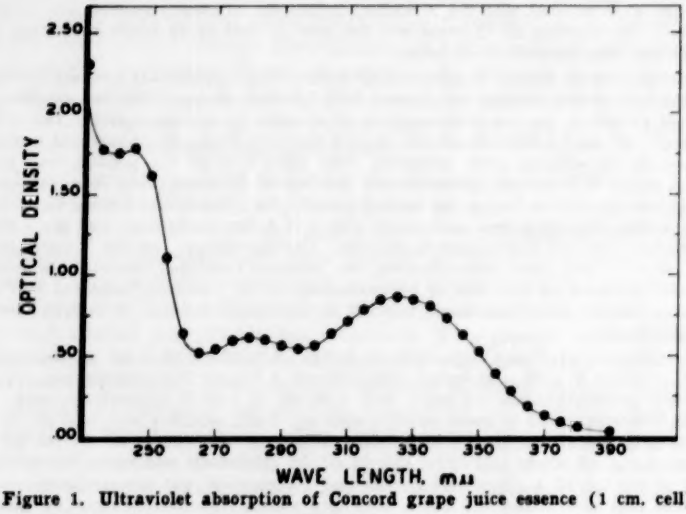

Figure 1. Ultraviolet absorption of Concord grape juice essence (1 cm. cell).

Synthetic essence. A synthetic essence was prepared with the composition shown in Table 1. Its specific gravity was 0.9932 at 20°C. (68°F.). The ultraviolet absorption spectrum was the same as that of the essence except that the weak maximum at 280 mμ was missing in the synthetic essence, and was replaced by a minimum at 270 mμ, optical density 0.14. The odor of the synthetic essence is discussed below.

RESULTS AND DISCUSSION

The results are summarized in Table 1.[c] As shown in this table the constituents which have been identified in Concord grape juice essence account for the total carbon content of the essence within experimental error. This indicates that no constituent present in sizeable amount has been overlooked. However, it would be entirely possible for the volatile constituents present in the greatest amount, and hence accounting for the

[c] The same volatile organic constituents were found in an essence prepared in the laboratory from 1950 Concord grape juice which had been prepared in a small tomato juice processing line *(13)* under conditions designed to minimize the possibility of chemical or microbiological changes during preparation.

major fraction of the carbon, to fail to account for the odor. This is true in the present instance since the odor of a synthetic essence prepared with the composition shown in Table 1 only slightly resembles the odor of the natural Concord grape juice essence. The identification of the volatile constituents is therefore incomplete.

It might be mentioned, however, that rarely has it been reported that the odor of a fruit is accounted for by the compounds which have been identified among its volatile constituents. In most instances no mention has been made of the odor of a mixture of the identified compounds, and it is likely that most identifications have been incomplete in this sense. In the present instance, it is known that the missing volatile constituents are present in the essence only in traces. Some information about this material has been obtained.

TABLE 1

Volatile constituents of Concord grape juice essence

Compound	Concentration, mg./ml.	Calculated millimoles carbon/ml.
Ethanol	35	1.52
Methanol	1.5	.05
Ethyl acetate	3.5	.16
Methyl acetate	0.15	.01
Acetone	0.3	.02
Acetaldehyde	0.03	....
Methyl anthranilate	0.033	....
Acetic acid	....[2]	....
Total		1.76[1]

[1] Total carbon found by combustion: 1.8$_1$ millimoles/ml.

[2] Acetic acid was absent from the essence but is present to the extent of approximately 0.03 mg./ml. in the vacuum distillate from grape juice.

If the Concord grape juice essence or the flash vacuum distillate from Concord grape juice is extracted with chloroform, and if the chloroform solution—after it has been washed with acid to remove methyl anthranilate—is evaporated, a small amount of oil is obtained. This oil is obtained to the extent of approximately 0.02 mg./ml., from the essence, and 0.004 mg./ml. from the vacuum distillate. If this oil is added at 0.02 mg./ml. to the synthetic essence, the odor of the mixture closely approximates the odor of the natural Concord grape juice essence. The composition of this oil is under investigation.

Data in Table 1 also lead to conclusions of interest in quality control work concerned with Concord grape juice. Approximately 90% of the volatile organic material in Concord grape juice is ethanol. Therefore, procedures for the quantitative determination of volatile matter by means of dichromate or permanganate oxidation *(9, 10)* if applied to Concord grape juice do little more than determine the amount of ethanol in the juice. Similarly, ethyl acetate and methyl acetate account for well over 95% of the volatile esters, and quantitative determinations of volatile esters in Concord grape juice do little more than determine ethyl and methyl acetate. In spite of their limitations, however, these procedures,

which determine the low-boiling volatile constituents, combined with the determination of a high boiling constituent such as methyl anthranilate probably furnish the best quality control procedure for volatile constituents that can be devised at present.

SUMMARY

A procedure is described for the identification of volatile constituents of fruits starting with as little as 10 kg. of material. Using this procedure, ethanol, methanol, ethyl acetate, methyl acetate, acetone, acetaldehyde, methyl anthranilate, and acetic acid have been identified as volatile constituents of Concord grape juice (*Vitis labruscana* Bailey). In addition to these compounds, the presence of at least one unidentified, chloroform-extractable constituent has been established. Conclusions of interest in quality control work are mentioned.

Acknowledgment

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STUDIES ON GROWTH AND TOXIN PRODUCTION OF *CLOSTRIDIUM BOTULINUM* IN A PRECOOKED FROZEN FOOD.

I. SOME FACTORS AFFECTING GROWTH AND TOXIN PRODUCTION[a]

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(Manuscript received February 21, 1955)

During recent years, the production of precooked frozen foods has rapidly increased *(2, 3)*, not only in total amount but also in variety. However, irrespective of this rapid increase, there has been little, if any, published information on the possibility of botulism from precooked frozen foods, although various investigators have studied the botulism problem in fresh frozen foods. The majority of the investigators feel that it is possible for botulinum toxin to develop in fresh frozen foods if such foods are grossly mishandled *(9, 13, 14, 15, 17, 19, 20, 21)*. Perry and her coworkers *(8)*, on the other hand, feel that this possibility is very remote.

Because precooked frozen foods are heated prior to the freezing operation, they may be considered as understerilized canned foods. Likewise, because *Clostridium botulinum* is widespread in nature, spores of this organism could be a common contaminant of the food being processed. The cooking treatment would be sufficient to destroy most, if not all, vegetative forms but may not be severe enough to destroy the spores of *Cl. botulinum*. Some investigators *(7, 8)* have found that the normal microflora of fresh frozen foods antagonizes the growth of *Cl. botulinum* and thus inhibits or lessens toxin production. Because the vegetative flora are largely destroyed in precooked frozen foods, this antagonistic action would be greatly reduced or eliminated.

Many precooked frozen foods are ideal growth media for *Cl. botulinum* and consequently if such foods are mishandled, e.g., left for an extended period at a growth temperature, they will probably become toxic. Since chicken a la king, a well-known precooked frozen food, contains all the necessary factors for growth of *Cl. botulinum*, it was chosen as the precooked frozen food for these studies. In the present paper, some of the factors thought to be important in relation to the growth and toxin production of *Cl. botulinum* in chicken a la king together with the possibility of the botulism hazard when such a precooked frozen food is mishandled are examined.

EXPERIMENTAL METHODS

Cl. botulinum (ATCC No. 7948–NCA strain 62A) was the organism selected for these studies. The medium used for preparing the spore stocks was a modification of that utilized by Reed *(10)*; casitone (Difco) was used instead of casein digested by the enzyme pancreatin. The inoculated media were placed in 250-ml. centrifuge bottles and incubated anaerobically in a desiccator for 7-10 days at 37°C. Anaerobiosis was

[a] This paper is a part of the thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Technology, University of Illinois, Urbana, Illinois, February, 1955.

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achieved by pyrogallic acid crystals and 20% Na_2CO_3. The spores were recovered by centrifugation, washed twice, suspended in M/20 phosphate buffer pH 7, and then kept under refrigeration after being heated for 15 minutes at 85°C.

Andersen's pea-pork medium *(1)* was used for counting both the *Cl. botulinum* spore suspensions and the total anaerobes as well as the anaerobic spores in the food filtrates. The medium contained 3% agar instead of the usual 1.5% to adapt the medium to the spin-tube technique *(6)*. In this technique 22- × 175-mm. tubes, each containing 12 ml. of medium, were used. For counting spores, the inoculated tube was heated to melt the agar, to destroy vegetative forms, and to heat activate the spores. Then 0.5 ml. of Seitz-filtered 5% $NaHCO_3$ were added to each tube to enhance spore germination. The cotton plug was pushed into the tube to within 3 inches of the bottom and the tube was then spun for 45 seconds. Anaerobic conditions were created in each tube by placing about one gram of pyrogallic acid and one ml. of 20% Na_2CO_3 in the upper portion of the tube, above the cotton stopper. The tube was plugged with a rubber stopper and incubated in an inverted position. Incubation was at 30°C. for 4 days.

Aerobic counts were made using tryptone glucose extract agar containing 0.5% yeast extract (TGEY). Incubation was at room temperature for 2 days.

Toxicity tests were performed on each food extract. A ratio of one part food (by weight) to one part distilled water (by volume) was found satisfactory for extraction; the food slurry was filtered, after 10 minutes of settling, through sterile cotton and cheesecloth. The filtrates were used for making bacterial counts, pH determinations, and toxicity tests. The latter were performed by interperitoneal injections of 0.2 ml. amounts of the clear filtrates (obtained by centrifugation) into white mice. The mice were observed for the development of botulinus symptoms for at least 5 days before the samples were recorded as positive or negative for botulinum toxin. Protection by antitoxin * confirmed the presence of botulinum toxin.

The formula for the chicken a la king prepared in this work was as follows: milk, 8 lbs.; chicken broth, 24 lbs.; chicken skin, 4 lbs. (cut into very tiny pieces); chicken fat, ¾ lb.; flour, 4½ lbs.; salt, ½ lb.; paprika, 6 grams; pimiento, 1¾ lbs. (canned, sliced on preparation); mushrooms, 2 lbs. (canned, sliced on preparation); frozen peas, 2 lbs. (defrosted on preparation); chicken ment, 10 lbs. (cut into ½-inch pieces); powdered onion, 2 oz.; powdered celery, 1 oz.

In this investigation four factors were studied: botulinum spore level, type of container, incubation temperature, and incubation period. Uninoculated controls were compared with two calculated levels of *Cl. botulinum* spore inoculum: a high level, with 4×10^3 spores per gram of food and a low level, with 4 spores per gram of food. Three types of containers were compared: carton (C), Frost-o-fold, with cellophane liner, no. 1 plain tin cans (T); and no. 1 C-enamel cans (E). The incubation temperatures of the defrosted samples were 50°F. (10°C.) and 86°F. (30°C.). The incubation periods were 2 and 5 days.

The chicken a la king was prepared and placed in the different containers in 9-ounce aliquots, and the containers (81 in number) were placed in a household type of refrigerator to cool (10°C.). The containers were then inoculated with the *Cl. botulinum* spores, which were thoroughly mixed into the food. The containers were sealed and placed in a deep freeze box (−10°F.), where they were stored for 20-36 days, after which time they were picked at random according to a predetermined scheme in which a random number was assigned to each sample. Nine samples representing the uninoculated controls, the two levels of inoculum and the three types of containers were defrosted and extracted directly without any incubation and were used as controls to demonstrate that the chicken a la king as prepared and handled was nontoxic. Of the remaining 72 samples, 36 were incubated at 50°F. (10°C.) and the other 36 at 86°F. (30°C.). At the end of the incubation period observations on gas formation, odor, and consistency of food were recorded, and the samples were then extracted. Bacterial counts, pH determinations, and toxicity tests were performed on each food extract.

RESULTS

Toxin formation. No toxin was produced during the freezing and thawing of the 9 samples that were defrosted and examined directly. Likewise, no toxin was detected

* Antitoxin type A and antitoxin type B were supplied through the courtesy of Lederle Laboratories, Pearl River, New York.

TABLE 1

Toxicity of the 36 samples incubated at 86°F. (30°C.)
Inoculum level of Cl. botulinum type A (spores/gram of food)

Incubation period in days	None						4						4×10^3						Total toxic samples
	C		T		E		C		T		E		C		T		E		
	Sample						Sample						Sample						
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	
2 days	−	−	−	−	+	−	−	+	−	−	−	+	+	+	+	+	+	+	9
5 days	−	−	+	+	−	−	+	+	+	+	−	−	+	+	+	+	+	+	12
Total toxic samples for each type of container	0		2		1		3		2		1		4		4		4		21

C = Carton container.
T = Plain tin container.
E = C-enamel container.
I, II = The two duplicates of each treatment.
− = Nontoxic sample (did not kill any of the injected mice).
+ = Toxic sample confirmed as having botulinum toxin (i.e., the two mice injected with the extract succumbed, whereas the third mouse which was protected with botulinum antitoxin survived).

TABLE 2

Final[1] pH values of the 36 samples incubated at 86°F. (30°C.)

Incubation period in days	Inoculum level of *Cl. botulinum* type A (spores/gram of food)																	
	None						4						4×10^3					
	C		T		E		C		T		E		C		T		E	
	Sample						Sample						Sample					
	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
2 days	4.90	4.95	5.30	5.00	5.00	4.90	5.87	5.31	4.84	5.10	4.93	4.95	5.50	5.55	5.27	4.85	5.63	4.90
5 days	4.89	4.93	5.15	4.82	4.82	5.12	5.60	5.40	4.83	4.89	4.97	4.55	6.25	5.37	4.85	5.72	6.03	4.92

[1] Original pH of freshly prepared chicken a la king was pH 6.5 ± 0.10.
Underlined figures are those of the toxic samples.
C = Carton container.
T = Plain tin container.
E = C-enamel container.

in any of the 36 samples incubated at 50°F. (10°C.) up to 5 days. At 86°F. (30°C.), however, certain of the 36 samples incubated at this temperature developed botulinum toxin (Table 1). Out of 36 samples, 21 proved toxic and were confirmed as having botulinum toxin. These 21 toxic samples included all (12/12) of the heavily inoculated samples, one-half (6/12) of the lightly inoculated samples, and one-fourth (3/12) of the uninoculated samples. The type of toxin in the inoculated samples was confirmed as type A. Results of the confirmation tests on the three uninoculated samples which proved to be toxic were not clear-cut. While the unprotected mice died with symptoms typical of botulism, protection was achieved by antitoxins of either type A or type B. When the data of these 36 samples incubated at 86°F. (30°C.) were analyzed statistically using the chi square method, the level of botulinum spore inoculum proved significant at the 0.001 level.

Out of the 21 toxic samples, 7 were from the carton containers, 8 from the plain tin cans, and 6 from the C-enamel cans. Statistical analysis showed a difference at the 0.03 level between the plain tin cans and the C-enamel cans in favor of the latter. There was, however, no significant difference between the carton containers and the metal containers.

Statistically, the effect of incubation period was not significant. The incubation temperature was highly significant because toxin did not develop in samples incubated at 50°F. (10°C.) but did develop in those incubated at 86°F. (30°C.).

pH changes. No detectable changes were observed in the pH of the samples examined after defrosting directly or those incubated at 50°F. (10°C.) up to 5 days. At 86°F. (30°C.), however, changes in pH occurred quickly, but to different levels, even after 2 days. The final pH of the 36 samples incubated at that temperature is shown in Table 2. Because the final pH of most of the toxic samples was higher than that of the nontoxic ones, it was thought that there might be a correlation between toxicity and the final pH of the samples. Analyses of the pH data using the procedure of "analysis of variance," the "F" test, showed that the botulinum spore inoculum was significant at the 5% level, that the effect of the type of container approached that level of significance, and that the incubation period had no significant effect on the final pH of the samples. The results of these analyses of variance are shown in Table 3.

TABLE 3

Analyses of variance for the final pH[1] of samples incubated at 86°F. (30°C.)

	Symbol	Degrees of freedom	Sum of squares	Mean square	Variance ratio	F	Level of significance
Level of inoculum...	L	2	1.1304	0.5652	4.64	0.05—	Significant at the 5% level
Type of container...	C	2	0.8239	0.4120	3.39	0.05+[2]	Approaches the 5% level
Period of incubation.....	P	1	0.0036	0.0036	0.03	N.S.	Not significant
Interactions.....	LC	4	0.8995	0.2249	1.85	N.S.	Not significant
	LP	2	0.2259	0.1130	0.93	N.S.	Not significant
	CP	2	0.0089	0.0044	0.036	N.S.	Not significant
	LCP	4	0.0180	0.0045	0.0371	N.S.	Not significant
Replications.....	.....	18	2.1899	0.1217	1	.....	.....
Total.....		35	5.3001				

[1] pH values of Table 2.
[2] The 5% level for 2 degrees of freedom is 3.55.

Changes in bacterial counts. The data for the bacterial counts of the samples of chicken a la king incubated at 50°F. and 86°F. for 2 and 5 days are collected in Table 4. At 86°F. (30°C.) a marked increase in total aerobic and anaerobic counts was observed in both the 2- and the 5-day samples, whereas a marked decrease in the spore counts was observed in the 2-day samples. This decrease was undoubtedly due to spore germination.

TABLE 4

The effect of incubation temperature and time on the bacterial counts [1] of samples of chicken a la king

Treatment after freezing Incubation temperature (°F.) Incubation period (days)	Inoculum level of *Cl. botulinum* type A (spores/gram of food)								
	None			4			4×10^3		
	Aerobic count	Anaerobic count		Aerobic count	Anaerobic count		Aerobic count	Anaerobic count	
		Total	Spores		Total	Spores		Total	Spores
	Organisms per ml. food extract								
No incubation	63	5	<1	$<10^3$	10	2	1.2×10^3	1.9×10^3	5.2×10^2
50°F. for 2 days	96	3	0	1.2×10^3	5	2	7×10^3	2.1×10^3	5.8×10^2
50°F. for 5 days	3.3×10^6	3.1×10^2	<1	1.7×10^7	1.4×10^3	1	3.1×10^6	1.5×10^3	3.3×10^2
86°F. for 2 days	1.5×10^7	4.3×10^6	<1	1.7×10^6	1.8×10^7	<1	8.3×10^7	2×10^7	6
86°F. for 5 days	3×10^7	7×10^6	14	2.9×10^5	1.7×10^7	5	3.4×10^7	1.3×10^6	2.8×10^6

[1] The geometric average of 6 samples, except for the nonincubated condition, where 3 samples were averaged.

Likewise, the higher spore count in the 5-day samples would most logically be the result of sporulation after multiplication.

Changes in odor and consistency of samples. No changes were detected in the odor of the samples incubated at 50°F. (10°C.) up to 5 days; the consistency of the 5-day samples, however, was less firm than that of those incubated for 2 days only. All samples incubated at 86°F. (30°C.) for 2 or 5 days had an off-odor and were spoiled. The toxic samples seemed to have peculiar odors (fecal, butyric, etc.). Some toxic samples, however, had only a slight off-odor of such a nature that it might not be objected to by some people.

DISCUSSION

Lack of toxin formation during freezing and thawing is in accord with the findings of most investigators of frozen foods *(4, 14)*. Likewise the lack of toxin formation in the samples incubated at 50°F. (10°C.) is in accord with the results of Edmondson *et al. (5)*, Straka and James *(13)*, Prescott and Geer *(9)*, and Tanner and Oglesby *(18)*. It has been demonstrated, however, that botulinum toxin may be produced, in certain cases, at comparable temperatures. Straka and James *(15)* found that one sample out of 83 uninoculated samples of frozen peas placed in cardboard containers and held at 50°F. for one week was slightly toxic. The pH was 5.3 and a mold was growing on the surface of the material. Another instance was reported by Tanner *et al. (17)*, who investigated toxin production in various inoculated frozen nonacid foods. At a 50°F. incubation temperature, 3 samples (two of spinach and one of ground beef) became toxic after 4 days. There were no visible signs of spoilage. The constancy of the anaerobic spore count of our samples which were incubated at 50°F. (10°C.) (Table 4) would imply that the spores were unable to germinate and/or to grow at this temperature and therefore would be unable to produce toxin.

The toxin formation at 86°F. (30°C.) was expected, for this is a favorable temperature. It was also expected that the botulinum spore level would be highly significant. The fact that 3 out of 12 uninoculated samples developed botulinum toxin emphasized the possibility of the botulism hazard in precooked frozen foods when such foods are mishandled. It is improbable that the uninoculated samples were inadvertently contaminated with the spore inoculum because these samples were sealed and placed in the freezer prior to the time of inoculating and handling the remaining samples. That mice were protected against the toxin of such samples by either type A or B antitoxin was confusing. This result, however, would imply that the type B monovalent antitoxic sera used contained enough A antitoxin to provide protection in samples of low toxicity or that a cross reaction took place.

The lower frequency of toxic samples in the C-enamel as compared to the plain tin containers is probably due to sampling results as well as other factors and not to an actual effect of the type of container on toxin production by *Cl. botulinum*. The differences in the total number of toxic samples came only from the lightly and the non-inoculated samples. In such samples, skips might be an important factor especially in view of the short incubation period. Also, the initial microflora could be more effective in inhibiting toxin production. The results were not consistent on the 2- and 5-day incubation periods and, in addition, other investigators have reported the opposite effect. Scott and Stewart *(11, 12)* found that

plain tin cans were more inhibitive to botulinum toxin production than were lacquered cans because of the tin ions released from the former. However, in a food as viscous as chicken a la king it is improbable that tin ions released from the tin containers would affect the organism within the mass of the food. The nonsignificant difference in toxicity between the carton containers and the metal containers confirms the findings of Perry and her co-workers *(8)*. These investigators found no evidence that hermetically sealed containers would introduce a public health hazard if used in the preservation of vegetables by freezing.

Changes in pH of all samples were associated with a considerable increase in all bacterial counts. The strain of *Cl. botulinum* used in this work possessed proteolytic properties. It would therefore be logical to expect an increase in pH whenever the botulinum organism has the upper hand in growth, whereas a lower pH would be expected if other organisms, especially the acid formers, gain predominance. The occurrence of some toxic samples with no abnormal appearance or objectionable odor emphasized the fact that obvious spoilage cannot be used as a safeguard against botulism.

SUMMARY

The following factors were studied for their effect on the growth and toxin production of *Cl. botulinum* (strain 62 type A) in precooked frozen chicken a la king after the food was defrosted: botulinum spore level, type of container, incubation temperature, and incubation period.

Botulinum toxin was found only in samples incubated at 86°F. (30°C.). No toxin was formed during the freezing and thawing operation or in samples incubated at 50°F. (10°C.). The effect of the size of the spore inoculum was statistically significant at the 0.001 level. There was no significant difference between the carton containers and the metal ones (plain tin or C-enamel), nor between the 2- and 5-day incubation periods.

Three of the 12 samples that received no botulinum spores contained botulinum toxin after incubation at 86°F. (30°C.). This emphasized the possibility of a botulism hazard when precooked frozen foods are mishandled.

The F value obtained from the analysis of variance of the final pH of the samples incubated at 86°F. (30°C.) indicated that the level of botulinum spore inoculum and the type of containers affected the pH.

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STUDIES ON GROWTH AND TOXIN PRODUCTION OF *CLOSTRIDIUM BOTULINUM* IN A PRECOOKED FROZEN FOOD

II. INHIBITION BY LACTIC ACID BACTERIA[a]

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(Manuscript received February 21, 1955)

It was demonstrated in a previous paper *(10)* that when detoxified spores of *Clostridium botulinum* were inoculated into chicken a la king, they would germinate, grow, and produce toxin when suitable environmental conditions were provided.

It would be desirable to establish conditions which would provide protection against this severe intoxication should such a food product be mishandled, i.e., should it inadvertently be allowed to defrost and remain at a growth temperature. There are three apparent possibilities for achieving such protection: the addition of a chemical inhibitor, the addition of an antibiotic, and the addition of an antagonistic microflora. Each method, however, has its own limitations. The use of a chemical inhibitor is limited by its own toxicity. The use of antibiotics in foods is currently not permitted in the United States. An antagonistic microflora must not be pathogenic or putrefactive and must be acceptable to the human senses. Lactic acid bacteria are acceptable on these counts, and, in addition, certain investigators have demonstrated that they possess an antagonistic action against the clostridia. Sherman *et al. (12)* reduced the potency of filtered botulinum toxin when either *Streptococcus lactis* or *Lactobacillus casei* were grown in it. Edmondson *et al. (3)* found that commercial raw milk inoculated with botulinum spores remained nontoxic because of the presence of lactic acid in such milk, while sterile and pasteurized milks became toxic. When they inoculated raw milk with a culture of *Streptococcus lactis* and only a few *Cl. botulinum* spores, curd formed and the cheese made from it was nontoxic. In recent years nisin-producing streptococci or a dried skim milk preparation of the antibiotic nisin, named "nisoplin," have been used in England and France in the making of processed cheese to inhibit gas-forming clostridia *(1, 5)*.

The work reported here was undertaken to gain information on the relative merits or shortcomings of various lactic acid cultures as the source of an antagonistic microflora which would suppress or delay growth and/or toxin production by *Cl. botulinum* in chicken a la king.

MATERIALS AND METHODS

The preparation of spore stocks, the media and method for counting anaerobic spores and total anaerobes, the method of extraction of food samples for pH determinations, for bacterial counts, and for toxin detection, and the method of preparing chicken a la king have been previously described *(10)*.

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[a] This paper is a part of the thesis submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Technology, University of Illinois, Urbana, Illinois, February, 1955.

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Tryptone glucose extract agar containing 0.5% yeast extract (TGEY) was found satisfactory for counting lactic acid bacteria as well as the total aerobic bacteria. Incubation was at room temperature for 2 days except when *Lactobacillus bulgaricus* cultures were used in the food sample, in which case the plates were incubated for 4 days. When a pure culture of this organism was counted, the incubation was at 37°C. for 2 days.

Three types of lactic acid bacteria were used in this study: *Strep lactis* X_{13}, nisin-producing strain,[c] a normal cheese culture[d] (a non-nisin-producing *Streptococcus* and several species of Leuconostoc), and *Lact. bulgaricus*.[d] These cultures were used in an active form by several daily transfers in sterile skim milk. A 16-hour culture incubated at 30°C. was used with the first two cultures. A 10-hour culture incubated at 37°C. was used in the case of *Lact. bulgaricus*.

In the last experiment, "nisoplin,"[e] a dried skim milk preparation of the antibiotic nisin, was used as one of the additives to the chicken a la king. It was added as a heavy slurry and was thoroughly mixed into the food sample in an amount which resulted in 160 Reading units of nisin per gram of food. This is the concentration recommended by the manufacturers for the inhibition of gas-forming clostridia.

RESULTS AND DISCUSSION

The effect of Strep. lactis X_{13} on toxin production by Cl. botulinum when grown in mixed culture. Thioglycollate medium (BBL) proved suitable for the growth of both *Cl. botulinum* and *Strep lactis* X_{13}. Twelve tubes, each containing 10 ml. of the thioglycollate medium of pH 7.3-7.5, were used in the experiment. Two tubes were left uninoculated and served as controls. The other ten, grouped in 2 sets of 5 tubes each, were inoculated with either *Cl. botulinum* spores or *Strep. lactis* X_{13} organisms, each alone or in combination as indicated in Table 1. One set of six tubes was incubated for 3 days and the other set for 10 days, both at 30°C. The pH and toxicity were determined on all samples. Bacterial counts were made on only the 10-day samples. The results of this experiment are shown in Table 1. The medium inoculated only with 0.2 ml. *Cl. botulinum* spores killed the mice after 1 and 2.5 hours, respectively, for the 3- and the 10-day incubation periods. The tubes, inoculated each with 0.2 ml. *Cl. botulinum* spores and 1 ml. *Strep. lactis* culture, were nontoxic after 3 or 10 days' incubation. The tubes having intermediate amounts of streptococci only delayed the death of the mice to times corresponding to the comparative ratio between the number of *Cl. botulinum* and the number of streptococci. The pH acted in a similar way; the toxin-producing organism kept the pH high, whereas the acid-producing streptococcus lowered the pH.

We may therefore conclude that *Strep. lactis* X_{13}, in the correct concentration, was able to antagonize *Cl. botulinum* and prevent toxin accumulation in the thioglycollate medium. The results of this experiment are in accord with the findings of Perry *et al. (9)*, who reported that one of the factors which determine whether a sample becomes toxic and to what extent it does so is the ratio of viable *Cl. botulinum* spores and cells to certain other bacteria in the sample.

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[c] Kindly supplied by Dr. N. J. Berridge, National Institute for Research in Dairying, University of Reading, England.

[d] Kindly supplied by Dr. S. L. Tuckey, Food Technology Department, University of Illinois, Urbana, Illinois.

[e] Kindly supplied by Aplin and Barrett, Ltd., Newton Road, Yeovil, Somerset, England.

TABLE 1

The antagonistic effect of Strep. lactis X_{13} on toxin production by Cl. botulinum when grown in mixed culture in fluid thioglycollate medium

Inoculum per tube, ml.		Incubation period in days at 30°C.	Final pH	Bacterial counts (per ml. medium)			Observations on injected mice
				Total aerobes	Anaerobes		
Cl. botulinum[1] spores	*Strep. lactis* X_{13}[2] organisms				Total	Spores	
0	0	3	7.52	0	0	0	Survived
0	0	10	7.30	0	0	0	Survived
0	0.2	3	4.92	—	—	—	Survived
0	0.2	10	5.06	2.5×10^9	2.7×10^9	0	Survived
0.2	0	3	6.94	—	—	—	Died after 1 hour due to botulism[3]
0.2	0	10	7.01	0	1.3×10^7	2×10^6	Died after 2.5 hours due to botulism
0.2	0.02	3	5.11	—	—	—	Died after 18 hours due to botulism
0.2	0.02	10	5.03	2×10^9	7×10^9	10^2	Died after 3 hours due to botulism
0.2	0.2	3	5.01	—	—	—	Survived
0.2	0.2	10	5.12	8.9×10^9	3×10^9	10^2	Died after 23 hours due to botulism
0.2	1	3	4.73	—	—	—	Survived
0.2	1	10	4.71	2.5×10^9	4×10^9	10^2	Survived

[1] *Cl. botulinum* spore stock—5×10^4 spores per ml.
[2] *Strep. lactis* X_{13} culture—10^3 organisms per ml.
[3] All deaths were confirmed as being due to type A botulinum toxin.

The inhibition of Cl. botulinum toxin production by lactic acid bacteria in "sterile" chicken a la king. The objectives of this experiment were to demonstrate the antagonistic action of lactic acid bacteria on toxin production of *Cl. botulinum* in chicken a la king without any interference from other microorganisms. *Salmonella* and/or *Shigella* organisms were isolated from most of the commercial samples of precooked frozen chicken a la king as well as from the other precooked frozen foods examined *(11)*. Because these organisms would have confused our toxicity tests, it was decided to work with a product as nearly sterile as possible in order to evaluate the effect of lactic acid bacteria on toxin production by *Cl. botulinum*. Accordingly, chicken a la king was prepared and packed in 96 baby food jars, each containing 50-g. aliquots. The jars were autoclaved for 15 minutes, at 15 lbs. steam pressure, and were allowed to cool in a household type refrigerator. The autoclaved food was practically sterile. One-third of the jars were left uninoculated, one-third received a light inoculum of 20 spores of *Cl. botulinum* per g. of food, and the last third received a heavy inoculum of 2×10^3 spores per g. of food. Each level of inoculum was divided into 4 groups of 8 jars each. The first group did not receive any lactic acid bacteria, the second received *Strep. lactis* X_{13}, the third received a mixed cheese culture, and the fourth received *Lact. bulgaricus*. In each case the inoculum was sufficient to provide a level of about 10^6 lactic acid

organisms per g. of food. The 96 jars were then divided into two equal parts. The first part was incubated directly (without being frozen) at 30°C. for 2 and 5 days. The second part was kept frozen for about one month before being incubated. After incubation, each sample was extracted, and bacterial counts, pH determinations, and toxicity tests were performed. Data on the results of the toxicity tests on the various incubated samples are presented in Table 2 and those of the final pH in Table 3. An inspection of the data presented indicates that the freezing and thawing of the samples was without effect on the subsequent results, and hence the results of these two parts of this experiment will be discussed together. The data, therefore, substantiated that the various lactic organisms can tolerate freezing, frozen storage, and subsequent thawing and are in accord with the results of Berry *(2)*, who found lactic acid bacteria to persist in frozen peas for at least 2 years when stored at −10°C.

The 16 samples inoculated only with *Cl. botulinum* spores were highly toxic; the average time for the death of the 32 mice injected with extracts from these samples was less than 1.5 hours. This would be expected because conditions for growth and toxin formation of *Cl. botulinum* were favorable and because the samples had been autoclaved they contained no microflora which would be antagonistic to *Cl. botulinum*. The incorporation of lactic acid bacteria into the samples was very effective in suppressing toxin formation. No toxin was detected in the 16 samples that contained both *Cl. botulinum* spores and *Strep. lactis* X_{13}. The use of the mixed cheese culture, however, resulted in one toxic sample, and the incorporation of *Lact. bulgaricus* resulted in 3 toxic samples. The 4 toxic samples in which the lactic acid bacteria failed to inhibit toxin formation were those which had received the larger inocula of botulinum spores and had been incubated for 5 days. The average time for the death of the mice injected with extracts from these samples was 6 hours. Statistical analysis showed that the average effect of the 3 cultures on toxin production by *Cl. botulinum* was highly significant; the probability that such results could be due to chance was less than 0.001. However, the small differences between cultures were not statistically significant. The fact that one of the samples which had received the mixed cheese culture and three of those which had received the *Lact. bulgaricus* culture were toxic might be attributed to inadequate mixing of the cultures into every part of the food sample. Since chicken a la king has a semi-solid consistency rather than a liquid consistency, an inadequate mixing of the culture might not permit complete exertion of the effect of the lactic acid bacteria throughout the whole mass of food.

The pH of the incubated samples dropped to pH 4.02-4.53 when either *Strep. lactis* X_{13} or the mixed cheese culture was added, and to as low as pH 3.43 when the *Lact. bulgaricus* culture was used. The pH of the uninoculated samples remained as high as that of the 16 toxic samples which had received botulinum spores only. Because of this, and because the pH of the 4 toxic samples in which the lactic acid bacteria failed to check botulinum toxin was as low as the pH of those which were not toxic, no simple correlation between toxicity and pH could be found.

TABLE 2

Effect of lactic acid bacteria on toxin production by Cl. botulinum in "sterile" chicken a la king incubated at 30°C.

Type of culture added	Treatment of samples before incubation	Inoculum level of *Cl. botulinum* type A (Spores/g. of food)												Total toxic samples	
		None				20				2×10^3					
		Incubation period (days)													
		2		5		2		5		2		5			
		a[1]	b[1]	a	b	a	b	a	b	a	b	a	b		
None	Not frozen	−*	−	−*	−	+	+	+	+	+	+	+	+	8	16
	Frozen[2]	−	−	−	−	+	+	+	+	+	+	+	+	8	
Strep. lactis X_{12}	Not frozen	−*	−	−	−	−*	−	−*	−	−*	−	−*	−*	0	0
	Frozen[2]	−	−	−	−	−	−	−	−	−	−	−	−	0	
Mixed cheese culture.	Not frozen	−*	−	−	−	−*	−	−*	−	−*	−	+	−*	1	1
	Frozen[2]	−	−	−	−	−	−	−	−	−	−	−	−	0	
Lact. bulgaricus	Not frozen	−*	−	−	−	−*	−	−*	−	−*	−	−*	+	1	3
	Frozen[2]	−	−	−	−	−	−	−	−	−	−	+	+	2	

Legend
− Nontoxic sample (did not kill or produce symptoms in the injected mouse).
+ Toxic sample (killed the two injected mice; the third mouse was protected by antitoxin type A); confirmed botulinum toxin type A.
* Nontoxic food extracts used later for detecting the mode of action of lactic acid bacteria.
[1] The letters a and b signify the two duplicates of each treatment.
[2] Samples frozen for one month before being incubated.

TABLE 3

Final pH values[1] of "sterile" chicken a la king inoculated with both Cl. botulinum spores and lactic acid bacteria and incubated at 30°C.

Type of culture added	Treatment of samples before incubation	Inoculum level of *Cl. botulinum* type A (spores/g. of food)											
		None				20				2×10^3			
		Incubation period (days)											
		2		5		2		5		2		5	
		a[2]	b[2]	a	b	a	b	a	b	a	b	a	b
None	Not frozen	6.22*	6.34	6.17*	6.15	6.26	6.08	6.36	6.35	6.18	6.02	6.11	6.13
	Frozen[3]	6.33	6.31	6.25	6.26	6.34	6.32	6.43	6.52	6.22	6.28	6.17	6.26
Strep. lactis X_{12}	Not frozen	4.22*	4.29	4.15	4.15	4.27*	4.30	4.21*	4.15	4.25*	4.28	4.02*	4.16*
	Frozen[3]	4.37	4.37	4.42	4.42	4.28	4.27	4.33	4.42	4.42	4.30	4.36	4.32
Mixed cheese culture	Not frozen	4.32*	4.27	4.37	4.35	4.40*	4.35	4.48*	4.46	4.44*	4.38	4.32	4.42*
	Frozen[3]	4.41	4.44	4.46	4.51	4.50	4.44	4.55	4.53	4.40	4.40	4.42	4.50
Lact. bulgaricus	Not frozen	3.68*	3.59	3.52	3.59	4.01*	3.65	3.70*	3.66	3.58*	3.50	3.43*	3.53
	Frozen[3]	3.61	3.74	3.73	3.88	3.65	3.66	3.70	3.92	3.52	3.75	3.73	3.77

Legend

Underlined figures are those of the toxic samples.

* The food extracts of these nontoxic samples were used later for detecting the mode of action of lactic acid bacteria.

[1] Original pH of freshly prepared sterilized chicken a la king was pH 6.5 ± 0.10.

[2] a and b are the two duplicates of each treatment.

[3] Samples frozen for one month before being incubated.

The uninoculated samples remained normal in odor and in consistency throughout the incubation period. No gas was observed in the food mass of any of the samples. This was to be expected, since the food was practically sterile to start with; the initial total aerobic count was less than 100 organisms per g. of food and reached only 7×10^2 organisms per g. of food after the 5-day incubation period. The 16 toxic samples which had received botulinum spores and no lactic culture had a peculiar odor which became predominantly fecal after the 5-day incubation period. The consistency of these samples was clearly changed even after the 2-day incubation period and was almost liquefied after 5 days. Cracks, due to gas formation in the food mass, were clear after 2 days' incubation. On the other hand, in the samples which had received lactic acid bacteria, a sour odor was generally noticed. The consistency of the samples that had received an inoculum of *Strep. lactis* X_{13} or the mixed cheese culture was normal up to the 5-day incubation period. Even the one toxic sample out of those which had received the cheese culture did not show any abnormal disintegration. This emphasizes that toxicity cannot be judged by appearance. After the 5-day incubation period the consistency of the samples that had received an inoculation of *Lact. bulgaricus* was somewhat more liquid than the control samples.

Possible modes of action of lactic acid bacteria against Cl. botulinum. Twenty nontoxic food extracts from the previous experiment were kept frozen until they were used in this experiment. Eighteen of these extracts are those shown with asterisks in Tables 2 and 3. The other 2 samples were controls that had received the heavy botulinum spore inoculum and either *Strep. lactis* X_{13} or the mixed cheese culture. The 2 samples were extracted directly without freezing or incubation to check the toxicity of samples before incubation. Thus the 20 nontoxic extracts represented 5 uninoculated samples, 6 lightly inoculated samples (20 spores per g. of food) and 9 heavily inoculated samples (2×10^3 spores per g. of food). The frozen extracts were allowed to defrost, after which they were neutralized to pH 7. They were then steamed for 10 minutes to kill all vegetative cells. Ten-ml. aliquots were transferred to sterile tubes, which were then sealed with a sterile petrolatum-wax mixture. The tubes were incubated at 30°C. for about one month, after which time odor, turbidity, gas formation under the seal, and pH were recorded. The contents of the tubes were centrifuged and the clear filtrates were checked for toxicity. Ten extracts were found to be toxic and were confirmed as having botulinum toxin. These were all the 9 extracts of the heavily inoculated samples and only one extract of the 6 lightly inoculated ones. None of the uninoculated samples became toxic. The pH dropped slightly to the ranges of 6.39-6.95. The toxicity was in all cases correlated with gas formation under the seal and a foul odor. All the 10 toxic samples were confirmed as having botulinum toxin type A, the same type as the inoculated spores.

The results of this experiment implied that the success of using lactic acid bacteria in the previous experiment was for the most part due to the acidity produced in the food by these bacteria, which inhibited the germi-

nation of *Cl. botulinum* spores and/or prevented growth of the germinated spores.

Other modes of action of lactic acid bacteria against *Cl. botulinum* are also possible. Sherman *et al. (12)* found *Lact. casei, Strep. lactis,* and *Proteus vulgaris* to have some ability to destroy filtered botulinum toxin, but the power of destruction they obtained cannot alone account for the results we obtained in our investigation for either *Strep. lactis* or *Lact. bulgaricus.* The *Strep. lactis* X_{13} used in our work is a nisin-producing organism. Hirsch *et al. (5)* obtained normal Swiss-type cheese when they used a starter of nisin-producing streptococci and milk heavily inoculated with *Cl. butylicum* spores, while the use of normal strain starter resulted in cheeses which were blown within 22 days. Similar results were obtained by Kooy and Pette *(7, 8).* Moreover, Hirsch *(6)* found that the lactobacilli do not produce the antibiotic nisin, but that they possess an inhibitory substance for *Cl. butylicum,* which was acid and heat labile and which was shown later by Wheater *et al. (17)* to be a peroxide. This substance might be of value in inhibiting *Cl. botulinum* as it has been proved to do against *Cl. butylicum.* The proteolytic ability of *Strep. lactis* has been studied extensively by Van der Zant and Nelson *(13, 14, 15, 16),* who related it to endocellular rather than exocellular enzymes. This proteolytic ability may account for some of the results obtained in our investigation.

The use of Strep. lactis X_{13} and "Nisoplin" in antagonizing toxin production by Cl. botulinum in chicken a la king of high initial microflora. It was decided, after the success obtained by using lactic acid bacteria against *Cl. botulinum* in sterile chicken a la king, to check the results when using a food of high initial microflora. The chicken a la king used in this experiment was a portion of that prepared for the sterile food experiment. After preparation, this portion was stored at 40°F. for 24 hours, and this slow cooling permitted the microflora to increase to a level of 10^6 organisms per g. of food without any appreciable changes in pH. Fifty-g. aliquots were placed in each of 36 baby food jars. Twelve jars were left uninoculated, 12 jars received a light inoculum (20 spores per g. of food), and the remaining 12 received a heavy inoculum (2×10^3 spores per g. of food). Each level of inoculum was divided into 3 groups of 4 jars each. The first received no lactic acid culture, the second received *Strep. lactis* X_{13} (4×10^6 streptococci per g. of food), and the third received a "nisoplin" solution (160 Reading units of nisin per g. of food). The 36 jars were then kept frozen for more than a month before being incubated at 30°C. for 2 and 5 days. The samples were extracted, and bacterial counts and pH and toxicity tests were made. The toxicity tests in this experiment were not clear-cut because certain of the food extracts caused illness and death in mice for causes other than botulinum toxin.

In the two-day incubation samples half of the samples inoculated only with botulinum spores were toxic but were not confirmed as having botulinum toxin. The use of nisoplin caused temporary ill effects even in the control samples that had had no botulinum spore inoculum. All the samples that had both botulinum spores and nisoplin were toxic but were not confirmed as having botulinum toxin. The incorporation of

Strep. lactis X_{13} in the samples, however, protected against any toxic or ill effect irrespective of the botulinum spore inoculum. In general, in the 2-day incubation samples no clear symptoms of botulism were observed in any case. The other symptoms accompanying the toxic cases that were not due to botulism were: fever, diarrhea, swelling of the eye lids, and general weakness. These symptoms were similar to those observed in previous work *(12)* where *Salmonella* and *Shigella* organisms were isolated from commercial chicken a la king as well as from other precooked frozen foods. These organisms, as well as others, were probably the cause of the toxic cases which were not confirmed as botulism. In these cases, extracts which originally caused fatal effects failed to do so when the tests were repeated using the same extracts after being frozen for several days. However, in most cases the mice receiving these extracts became obviously ill but recovered. Likewise, the antitoxin appeared to have no protective effect.

In the 5-day incubation samples, however, different results were obtained. Five of the 18 extracts when tested for toxicity produced typical symptoms of botulism in the mice. In three of the five cases, antitoxin type A gave complete protection, in the other two cases it delayed the death of the mice which died with symptoms other than botulism. These 5 toxic samples were as follows: one of the 2 samples with the heavy botulinum spore inoculum and no additive; the 2 samples with both the heavy botulinum inoculum and *Strep. lactis* X_{13}; and 2 of the samples with the nisoplin, one of which had received the light, the other the heavy botulinum spore inoculum. In general, all the 5-day samples that contained *Strep. lactis* X_{13} were toxic because of botulinum toxin and/or other pathogenic organisms irrespective of the level of botulinum inoculum. There seems to have been a synergistic action between the streptococci added and the pathogenic organisms that were likely to be present. It is apparent also that the incubation period and temperature had a major effect as half of the samples that received no additives were toxic after the 5-day incubation at 30°C. whether they had been inoculated with botulinum spores or not.

The use of "nisoplin" seemed not to be of beneficial effect against *Cl. botulinum* or other organisms under the conditions of our experiment. One-half of the samples that contained nisoplin were toxic and their extracts killed the mice whether the samples had received a botulinum inoculum or not. Even the gas formers were not inhibited by the nisoplin. This might be attributed to the fact that certain acid producing organisms have been found *(4)* which nullify the effect of the antibiotic nisin, the active ingredient in nisoplin. This was explained as being due to a synergistic action between the nisin and the gas producing clostridia. These conditions were probably present in our experiment where we created a food of high initial microflora, some of which probably were acid formers of the types which would cause this synergistic action to take place. If we had started with food having a low total bacterial count, such as we would obtain under excellent processing conditions, these acid formers probably would have been at a minimum, and the nisin would

have been able to exert its action on the gas-forming clostridia as the product "nisoplin" is intended to do.

SUMMARY AND CONCLUSIONS

In a preliminary experiment in which fluid thioglycollate medium was used, *Strep. lactis* X_{13} was able, in the correct concentration, to inhibit the accumulation of botulinum toxin in the mixed culture medium.

The work with chicken a la king included a series of experiments in which several types of lactic acid bacteria were checked for their ability to inhibit the formation of *Cl. botulinum* toxin in chicken a la king of different initial bacterial counts. The results may be summarized as follows: Three lactic acid starters were used in "sterile" chicken a la king against different levels of botulinum spore inoculum. Even after a 5-day incubation at 30°C., none of the 16 samples that had received both an inoculum of *Strep. lactis* X_{13} (a nisin-producing Streptococcus) and botulinum spores became toxic. Only one of the 16 samples that had had both botulinum spores and the mixed cheese culture was toxic. In the case of *Lact. bulgaricus* three of the 16 samples were toxic. Statistical analysis showed no significant difference between the effects of the three lactic cultures. Their average effect against botulism hazard was significant at the 0.001 level. The use of *Lact. bulgaricus,* however, is not recommended, because a considerable proportion of samples proved to be toxic, nor can the other cultures be recommended as giving complete protection until more tests have been performed.

The effect of lactic acid bacteria against the formation of *Cl. botulinum* toxin is for the most part sporostatic; spore germination was inhibited by the acidity of the lactic acid which is formed from the fermentation of the food sugars. Other modes of action are also discussed.

The use of *Strep. lactis* X_{13} in chicken a la king of high initial microflora protected against botulism hazard as well as against the ill effects from other organisms in the samples incubated at 30°C. for 2 days but not in those incubated for 5 days. The use of "nisoplin" in the same experiment was not beneficial even after the 2-day incubation period. It also failed to check gas formers because of the presence of the high initial microflora.

We may generally conclude that the introduction of lactic acid bacteria into precooked frozen foods, such as chicken a la king, offers considerable protection against the development of toxin by *Cl. botulinum* in such foods. This procedure would be most effective in foods which had been produced under the most desirable processing conditions. However, more work is needed to determine whether any of the lactic acid bacteria would completely inhibit toxin formation by *Cl. botulinum* and also to adapt the use of lactic acid bacteria to practical applications.

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STUDIES ON HIGH TEMPERATURE STERILIZATION OF EVAPORATED MILK WITH EMPHASIS ON STABILITY ASPECTS*

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There are interesting problems related to every type of food in the development of high-short processing. None is more interesting and puzzling than the problems pertaining to milk, especially evaporated milk. High-short sterilization can be used on fluid milk and dairy drinks as well as on evaporated milk. In the study to be described, however, foremost attention was given to evaporated milk for the following reasons:

a. From a canning standpoint, evaporated milk is more attractive economically than fluid milk.

b. A large percentage of the problems that pertain to processing dairy products is found in evaporated milk production.

c. In this study, reconstituted high-short processed evaporated milk was generally found to have better flavor than high-short processed fluid milk, coming from the same raw material.

Much was learned about the inherent physical instability of evaporated milk—both that which results in cream separation and that which produces coagulation of the product. Physical instability of the types just mentioned is commonly found in milk that has been subjected to a high-short sterilizing process because a short heat treatment fails to impart to milk certain properties of texture stability which are imparted by a process of conventional length.

Many factors were examined in this study in an effort to make stability with respect to cream separation compatible with high-short processing. These factors included forewarming treatment, chemical correctives, increase in solids content, turbulence conditions in the products before, during, and after processing, and the addition of colloidal stabilizers. Of these five factors, the last named—i.e., the addition of colloidal stabilizers—brought greater benefit to stability than any other.

Sterile evaporated milk produced by a high-short process in which the milk is completely sterilized, or almost completely sterilized, before it is put into the can is equal to practically every demand as a substitute for pasteurized milk.

Without the use of physical stabilizers, the shelf life of heat sterilized evaporated milk, which is of such quality as to be acceptable for beverage purposes, is only one or two months at room temperature. With the use

* Acknowledgment is made to the American Can Company and to the Research Department of that organization whose kind permission to use the results of experimental work referred to in this paper made possible the present report.

of a stabilizing material, the shelf life may be increased to approximate that of evaporated milk processed by the usual long-hold method. Nevertheless, this treatment does not necessarily dispose of the difficulties inherent in high-short sterilization. Stabilizing substances of colloid nature must be used cautiously. As a rule, they lower the coagulating point of the product, making it more difficult to sterilize without producing coagulation. With some milk, this constitutes a real problem.

Another question must be considered in deciding upon the advisability of using stabilizing ingredients. Due to unexplained causes, high-short sterilized milk does not retain its original flavor in storage for an indefinite period. Chemical reactions of a non-enzymic nature may be responsible for the change in flavor. Oxidation is a well known cause of flavor deterioration in milk, but the possibility of preventing development of off flavor in canned milk by removal of oxygen from the product has not been demonstrated. It is of doubtful advantage to take measures to extend texture stability beyond the period of flavor stability. As stated earlier, flavor stability in evaporated milk of flavor quality similar to that of pasteurized milk lasts for only a few months under ordinary conditions. Either with or without the use of physical stabilizing materials, it may be advisable to control the distribution of this product to ensure its consumption within the period of its flavor stability. The stable life of high-short sterilized milk, from the standpoints of both texture and flavor, can be prolonged for several months by storing the product under refrigeration.

Scope of this report. The investigation covered by the data in this report dealt not with colloidal stabilizers but with two of the other four stabilizing factors mentioned above; namely, chemical correctives and variation in solids content of the milk. The first part of the report treats of means of preventing coagulation of evaporated milk while producing satisfactory viscosity during sterilization by a high-temperature short-time method. The second part deals with questions pertaining to the adequate sterilization of the product and to stability in storage following sterilization by various procedures.

Beneficial results in controlling coagulation and viscosity were obtained by the use of chemical correctives of both acidic and alkaline nature. These results are significant in respect to heat sterilization of evaporated milk; they are especially pertinent to high-temperature short-time methods, as previously pointed out by the author *(2)*. The results will be presented in graphs, showing the relationships between quantities of lactic acid used and the amounts of coagulation and viscosity produced in the product during processing.

Simultaneously with the study of stability, valuable information was accumulated in the use of end-over-end rotation of cans in sterilizing evaporated milk. The use of acid as a corrective had previously been studied with evaporated milk processed by so-called aseptic methods, and it was learned that the same principles pertaining to the use of acid apply to those methods as to agitated can methods of processing. These principles will be elucidated as they were developed in agitated can processing, since a more extensive study of interrelated factors was made with this method than with aseptic canning methods.

PART I—VISCOSITY AND COAGULATION CONTROL

Milk used in these experiments was of the grade commonly used in condenseries. It was prepared as evaporated milk by standard procedures of forewarming and evaporating. The raw material for the different batches had the usual variations in the properties which are manifested by a tendency to coagulate when heated. Except for the addition of corrective substances and for differences in degree of concentration, the preparatory processing of all batches was identical. The details of the forewarming and evaporating treatments are of no importance; therefore, this report will not be burdened with them. Distinctions among batches and lots, insofar as results are concerned, are accounted for solely by (a) variations in inherent properties of the raw milk, (b) variations in the degree of concentration of the product (ca. 26% or ca. 32% total solids), and (c) the variations in corrective and sterilizing treatments which constituted the foundation of the experiments. All studies were made with milk that was heated for sterilization indirectly, that is, without the introduction of live steam into the milk.

The assortment of blends used may seem somewhat involved. The value of the data, however, is embodied in the blending aspect of the treatment with correctives.

EXPERIMENTAL

Processing equipment. An experimental Fort Wayne sterilizer with a specially designed reel holding 78 sanitary type cans of 301 × 403 size was used. The speed of rotation was variable between 44 and 82 r.p.m. Coming-up time of one minute was

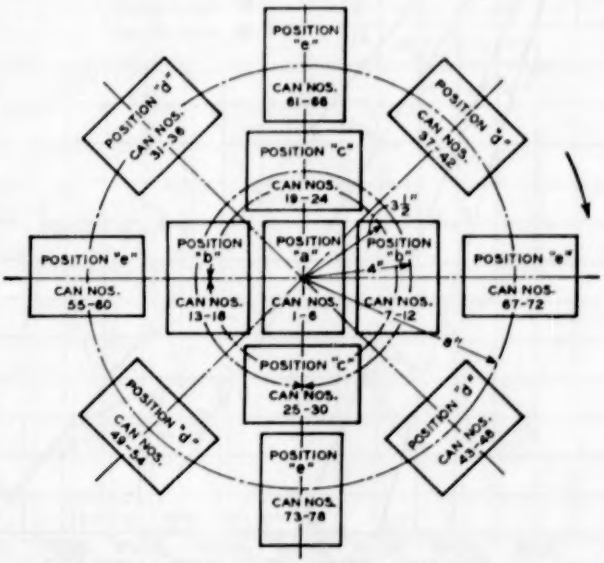

Figure 1. Diagram showing the position of cans in the rotating reel in the experimental sterilizer. Front view shown. Cans were placed six deep, numbered from front to rear. Direction of rotation: clock-wise.

standard for the experiments. Cans of each batch were numbered consecutively from 1 to 78 to identify them exactly with respect to position in the reel. A front view plan of the reel is shown in Figure 1.

The amount of agitation each can received depended upon which one of the 5 positions, *a*, *b*, *c*, *d*, or *e*, the can occupied. When the reel was filled, there were 6 cans in every row. The farther away from the axis of rotation a can was, the less benefit its contents received in the way of increased agitation when the speed of rotation was increased. The orientation of the can, with respect to the axis of rotation, also had an influence on the relation between speed and agitation. For example, the center of a can in position *c* was the same distance, 8 inches, from the axis of rotation as was the center of a can in position *d*. Nevertheless, can *d* received slightly more benefit from increased rotation speed than can *c* because the principal axis of the former was tangent to the path of travel of the center of the can while the principal axis of the latter was perpendicular to this tangent. Centrifugal force had slightly less effect on the product in can *d* than in can *c*.

For a can in either position, *d* or *e*, it is easily calculated that, assuming the mass of its contents to be concentrated in the center point of the can, centrifugal force on

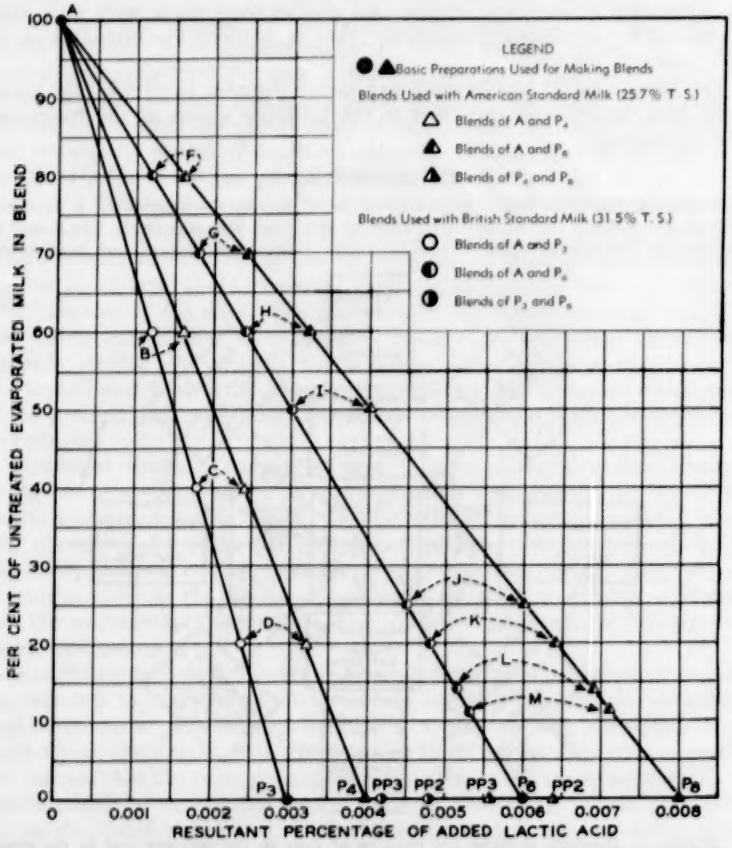

Figure 2. Preparations used in evaporated milk blending experiments excluding alkaline correction.

this mass is greater than gravitational force when the speed of rotation is greater than 66.3 r.p.m. From a study of Figure 1, one sees clearly why the effect of the gravitational component, which is relatively weaker than the centrifugal component of the forces acting on the milk at higher speeds, would result in somewhat less agitation in can *c* than in can *d*. The milk in the extreme outward portion of can *c* is more affected by centrifugal force than any portion of the milk in can *d*. It is therefore less free to respond to gravitational force than is the milk in can *d*.

The contest between centrifugal force which tends to prevent agitation and the force of gravity which tends to produce agitation veers more and more to the side of gravity in each individual can the nearer the can is placed to the axis of rotation. Thus, the ideal condition from the standpoint of agitation is obtained in position *a*, which is centered on the axis of rotation.

Procedure and results of blending experiments. In the blending experiments, milk that had been corrected to the acid side was blended with milk that had been corrected to the alkaline side as well as with milk that had not been corrected. It was found that, with most types of raw milk having a light forewarming treatment, the chemical balance that produced the maximum elevation in coagulation point (meaning reduction in

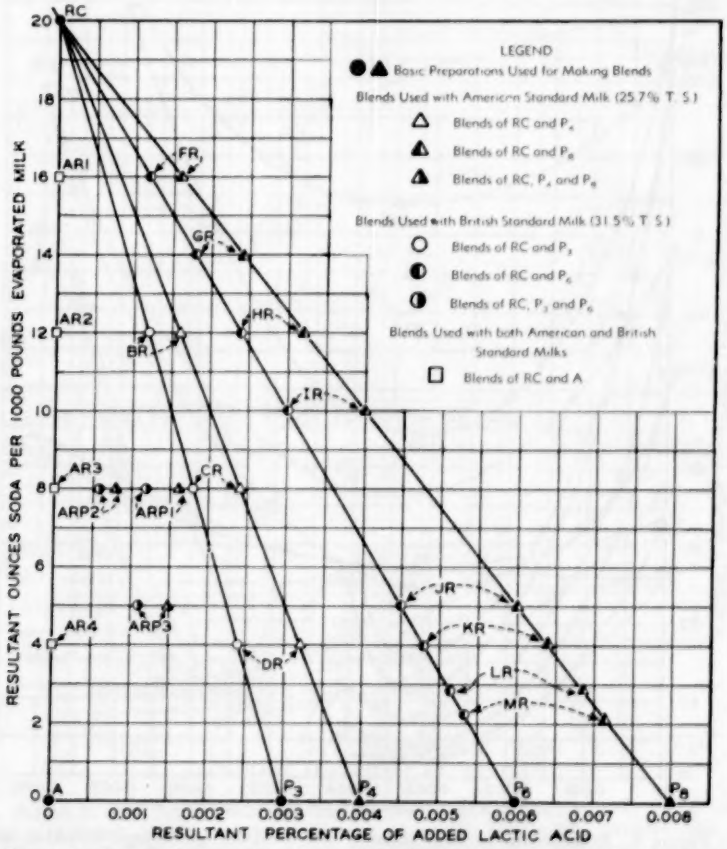

Figure 3. Preparations used in evaporated milk blending experiments—blends containing alkaline (soda) correction.

coagulation with a given process) was obtained in a blend of milk that was corrected to the alkaline side with milk that was corrected to the acid side. With certain types of processing, good results were obtained by blending milk that was corrected to the acid side with milk that was not corrected. The 6 basic preparations used in the blending experiments consisted of non-treated evaporated milk, lot A, and 5 treated lots, designated as P_3, P_4, P_6, P_8, and RC, respectively. The treated lots were prepared by adding lactic acid or soda[b] to the raw milk before evaporating. Those containing acid, together with the blends of those lots, are designated by plotted points in Figure 2 which show the percentages of untreated evaporated milk (lot A), as well as the

[b] It should be noted that U. S. Federal Regulations do not permit the commercial use of sodium bicarbonate as a corrective agent for evaporated milk. This substance was used in these experiments to illustrate the results of correction on the alkaline side for reasons of convenience, also, because there are records of a considerable amount of experience back of the use of sodium bicarbonate as a corrective.

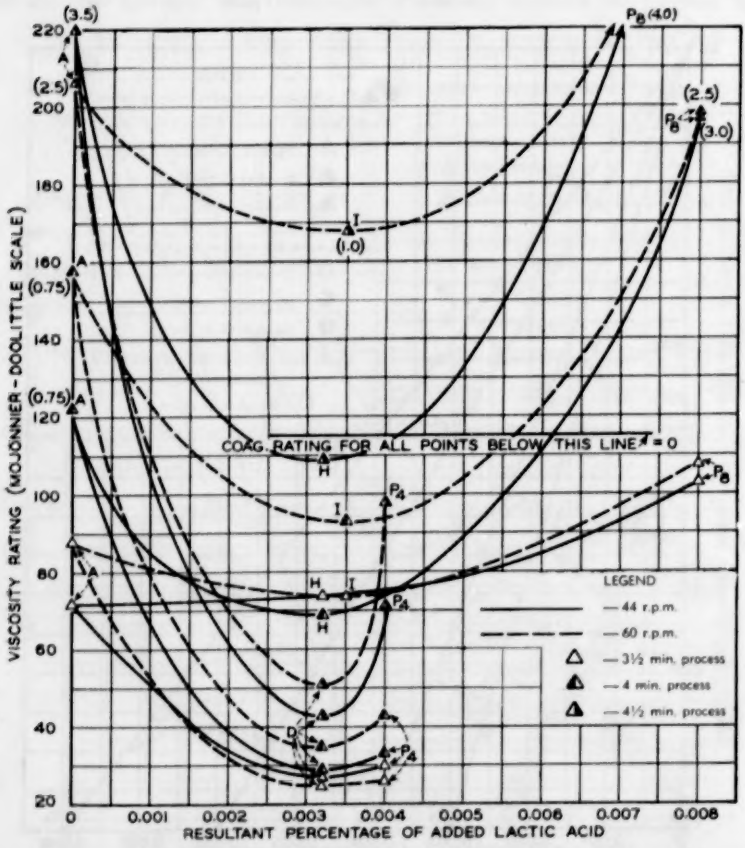

Figure 4. Relationship between viscosity and resultant percentage of added lactic acid in different blends of evaporated milk of 25.7% total solids content—code 12. Process at 260°F. (Numerals adjacent to plotted points are ratings for coagulation.)

resultant percentages of added acid in the blends. Figure 3 shows the blends of which lot RC was a component. This figure shows also the resultant quantities of added soda and acid in the blends.

Sanitary 301 × 403 cans were used in the experiments. Cans containing the various mixtures were processed while being turned end over end at the rate of 60 or 44 r.p.m. The cans were placed in position *e* (centers *were 8 inches from the axis* of rotation).

In each process, one minute was consumed in bringing the temperature of the sterilizer to the holding temperature of 260° or 270°F. (126.7° or 132.2°C.).

Cooling was accomplished by spraying cold water on the cans immediately after the termination of the holding time and permitting the sterilizer to fill with water as rapidly as possible. Then the water was allowed to flow through the sterilizer, discharging through a vent in the top. The movement of the cans at the rate of 60 or of 44 r.p.m. was continued until cooling was completed.

The samples were examined on the day on which they were processed.

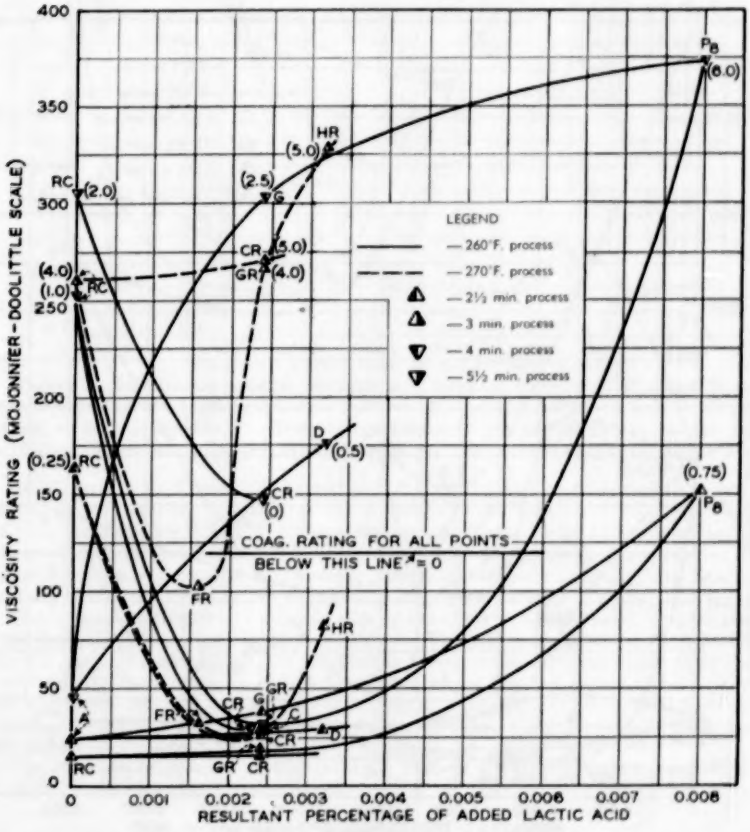

Figure 5. Relationship between viscosity and resultant percentage of added lactic acid in different blends of evaporated milk of 25.7% total solids content—code 16. Process at 60 R.P.M. (Numerals adjacent to plotted points are ratings for coagulation. Preparation RC had 20 ounces soda per 1000 pounds evaporated milk.)

The rating for viscosity is in terms of degrees retardation on the Mojonnier-Doolittle viscosimeter, using the medium size ball.

The rating for coagulation is in terms of an arbitrary scale which includes the following designations: 0, .25, .50, .75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0. The rating, 0, designates perfectly smooth texture, with no trace of coagulation. The rating 1.0, designates slight coagulation, manifested by the appearance of numerous specks of coagulum on a black rubber spatula after it has been dipped into the milk and drained. Coagulation having a rating of 1.0 is the maximum that can be tolerated in evaporated milk on the commercial market. Ratings between 0 and 1.0 designate conditions intermediate to freedom from coagulation and the maximum coagulation that is tolerated in commercial milk. The rating, 6.0, designates complete coagulation. Ratings between 1.0 and 6.0 designate conditions intermediate to the maximum coagulation tolerated in commercial milk and complete coagulation.

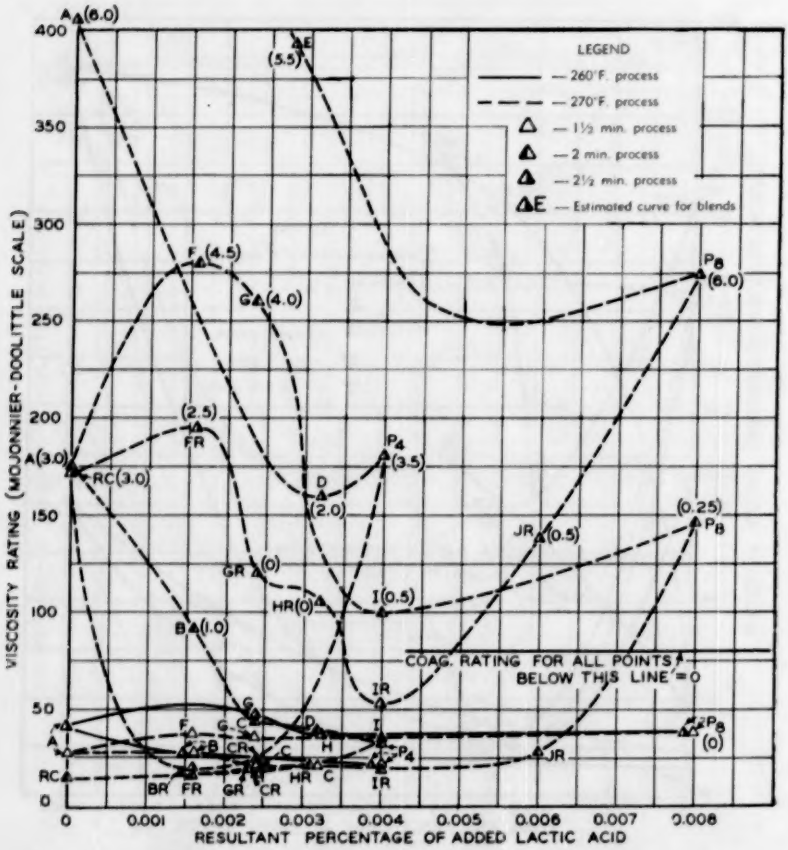

Figure 6. Relationship between viscosity and resultant percentage of added lactic acid in different blends of evaporated milk of 25.7% total solids content—code 17. Process at 60 R.P.M. (Numerals adjacent to plotted points are ratings for coagulation. Preparation RC had 20 ounces soda per 1000 pounds evaporated milk.)

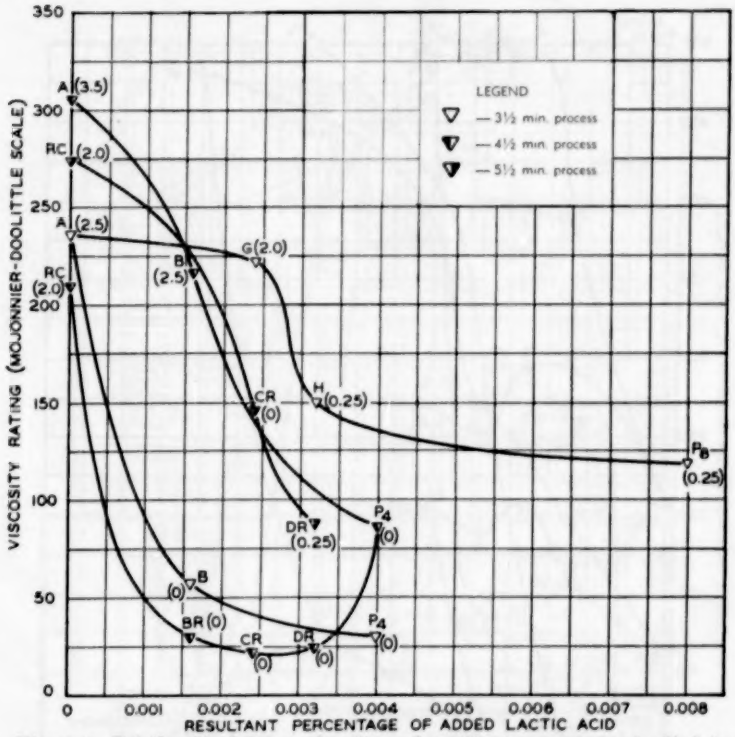

Figure 7. Relationship between viscosity and resultant percentage of added lactic acid in different blends of evaporated milk of 25.7% total solids content—code 17. Process at 60 R.P.M.—260°F. (Numerals adjacent to plotted points are ratings for coagulation. Preparation RC had 20 ounces soda per 1000 pounds evaporated milk.)

In Figures 4, 5, 6, and 7, coagulation and viscosity data from experiments with milk of 25.7% total solids are given for processes at 260° and 270°F. (126.7° and 132.2°C.) and Figures 8 and 9 give similar data for the milk of 31.5% total solids content. Four different batches of raw milk are represented—one in Figure 4, one in Figure 5, one in Figures 6 and 7, and the fourth in Figures 8 and 9. These batches are designated respectively by the code numbers 12, 16, 17, and 24.

The effect of increments of time, as well as the effect of difference of temperature, in the sterilizing processes is shown in the figures. Viscosity is indicated by the ordinate scale for the curves and ratings for coagulation are shown as numerals adjacent to the plotted points. In addition, in Figure 8, adjacent to the four AR points and the three ARP points are indicated the resultant quantities of added soda in ounces per thousand pounds of evaporated milk. Preparation RC, in all cases, had 20 ounces of soda per 1000 pounds of evaporated milk.

The letter designations of plotted points in Figures 4 to 9 indicate the constitution of the blends as shown on Figures 2 and 3.

DISCUSSION

A wide variation in degrees of inherent stability against heat was demonstrated among the 4 batches of milk. This is shown in the graphs by ratings for coagulation and viscosity of the lots A of the different batches.

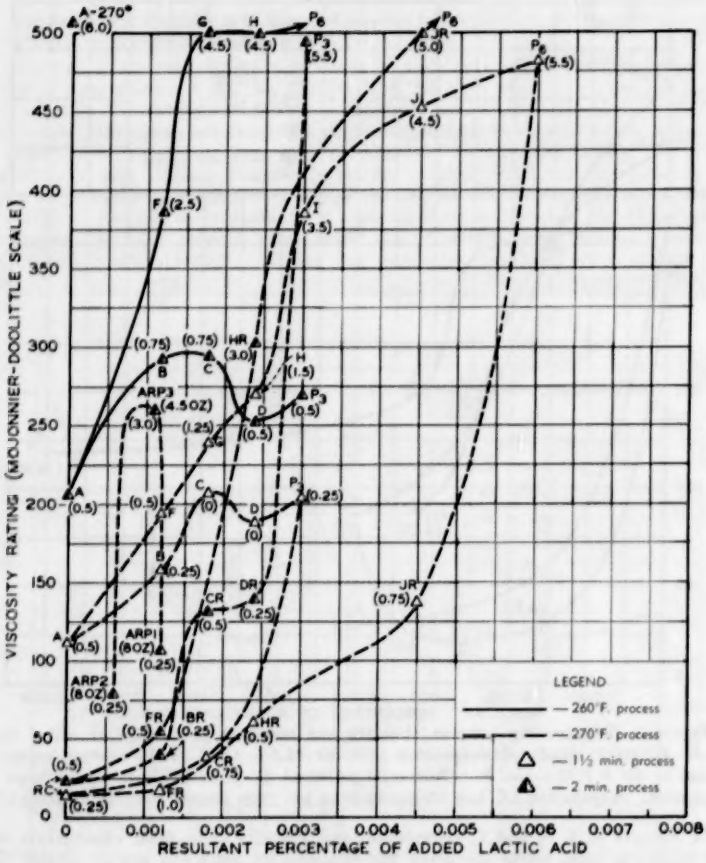

Figure 8. Relationship between viscosity and resultant percentage of added lactic acid in different blends of evaporated milk at 31.5% total solids content—code 24. Process at 60 R.P.M. (Numerals adjacent to plotted points are ratings for coagulation; in addition, adjacent to ARP points are indicated the resultant quantities of added soda in ounces per 1000 pounds evaporated milk. Preparation RC had 20 ounces soda per 1000 pounds evaporated milk.)

The graphs show that the percentage of added acid is not a criterion of the intensity of the effects produced by the addition of acid upon the coagulation point and the viscosity of the milk. These effects depend largely upon the manner in which the acid is added as well as on the processing conditions and the inherent chemical properties of the milk. If we consider several blends, each of which contains 0.003% of added acid, the curves show that the viscosity produced depends upon whether the blend is produced by using treated lot P_3, P_4, P_6, or P_8. Table 1 shows these variations for a number of blends of batches 12, 17, and 24, in which the lots designated

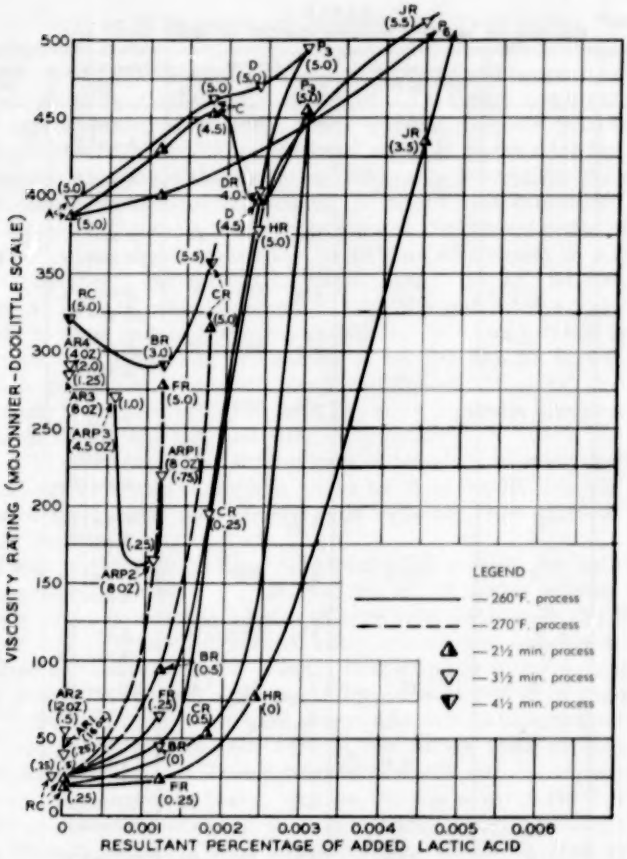

Figure 9. Relationship between viscosity and resultant percentage of added lactic acid in different blends of evaporated milk of 31.5% total solids content—code 24. Process at 60 R.P.M. (Numerals adjacent to plotted points are ratings for coagulation; in addition, adjacent to AR and ARP points are indicated the resultant quantities of added soda in ounces per 1000 pounds evaporated milk. Preparation RC had 20 ounces soda per 1000 pounds evaporated milk.)

A were used. For convenience in later discussion, examples like those listed in Table 1 are said to have "large difference" characteristics.

Higher ratings are invariably obtained in blends employing lot P_8 than in blends employing P_4 and in blends employing P_6 than in blends employing lot P_3.

The blending of acidified lots with sodium bicarbonate treated (RC) lots produced, for all processes on 25.7% solids milk covered in this investigation, a reduction in ratings for coagulation and viscosity from those for the RC lots alone. In certain processes, comparatively great differences in coagulation and viscosity ratings, for given resultant percentages of added

TABLE 1
Viscosity of blends containing 0.003% of added lactic acid

Code	Figure	Concentration (percent solids)	Process			Components of blend	Viscosity rating
			Min.	°F.	RPM		
12	4	25.7	3½	260	44	A and P_4	27
						A and P_8	74
			4	260	44	A and P_4	29
						A and P_8	69
			4½	260	44	A and P_4	44
						A and P_8	109
			3½	260	60	A and P_4	25
						A and P_8	75
			4	260	60	A and P_4	35
						A and P_8	94
			4½	260	60	A and P_4	51
						A and P_8	168
17	6	25.7	2	270	60	A and P_4	40
						A and P_8	160
17	7	25.7	3½	260	60	A and P_4	35
						A and P_8	160
24	8	31.5	2	260	60	A and P_3	270
						A and P_8	510
			1½	270	60	A and P_3	205
						A and P_8	385
16	5	25.7	4	260	60	RC and P_3	35
						A and P_8	320
17	6	25.7	2½	270	60	RC and P_3	112
						A and P_8	376
			2½	270	60	RC and P_4	50
						RC and P_8	112
24	9	31.5	2½	260	60	RC and P_8	150
						A and P_8	445

lactic acid, are shown for blends of lot RC with lots P_4 and P_8. Instances of this condition, applied to products under codes 16 and 17 containing 0.003% of added acid, are shown in Table 1.

Some examples presented in the graphs show little or no difference in coagulation and viscosity ratings for given final percentages of added acid, due to the selection of certain lots in making the blends. We may refer to these as "examples of small difference." The inherent stability characteristics of the milk seemed to control this attribute. No instance of this kind appeared in batch 12 (Figure 4). In other words, every batch 12 example was an "example of large difference." It is noteworthy also that, since batch 12 had a high degree of inherent stability, alkaline corrective did not figure in the treatment of this milk. In batch 12, an increase in acid invariably increased the stability (caused reduced viscosity) until added acid equalled 0.003%. A further increase in acid produced an increase in

viscosity. Increase in agitation also produced generally higher viscosity in blends A, D, and P_4.

Batch 16 (Figure 5) gave satisfactory viscosity and coagulation ratings in blends containing either lot A or lot RC. This batch apparently had a higher degree of inherent stability (lower original viscosity) than batch 12; however, it reacted very differently from batch 12 to the addition of acid. The coagulating point of batch 16 was noticeably lowered by the addition of acid; thus, it was advantageous to use an alkaline corrective. Blends containing lot A showed a tendency to assume the characteristic of "large difference," while blends containing lot RC tended strongly to assume the "small difference" characteristic. Even though the RC lot had higher viscosity and lower coagulating point than the untreated lot A, since a moderate increase in acid content generally produced an increase in coagulation point and a decrease in viscosity of lot RC, this lot served as well as lot A in producing blends with good stability and viscosity. Increase in temperature from 260° to 270°F. in a 2½-minute process produced higher viscosity in blends RC, CR, and GR.

Batch 17 (Figures 6 and 7) also gave satisfactory viscosity and coagulation ratings in blends containing either lot A or lot RC but the characteristics of the various blends were quite different from those of similar blends of batch 16.

It is well known that the higher the total solids content, the less resistant is evaporated milk to heat. Figures 8 and 9 are of special interest because they show data representing a batch of high total solids milk (31.5% total solids) of especially low inherent stability against heat—so low that only an alkaline corrective makes it possible to sterilize it without coagulation. The uncorrected milk stood a process of 2 minutes at 260°F. or 1½ minutes at 270°F. but neither of these processes is adequate to ensure sterilization of the product. Every addition of acid, no matter what procedure was followed, lowered the coagulating point of the milk (produced coagulation at reduced temperature or time); thus lot RC was more stable than either lot A or any blend containing acid—a condition that practically never exists with milk of 25.7% total solids content. In the shortest processes which were adequate for sterilization, blends of 31.5% solids milk containing either lot A or lot RC gave satisfactory viscosity and coagulation ratings.

Milk to which acid was added after evaporation was not included in this study. It had been learned previously that the addition of acid after evaporation lowers the coagulating point without giving desired increase in viscosity.

It is felt that there is a sufficient degree of consistency in the data presented above to make these data capable of serving as a guide to the establishment of a chemical treatment of evaporated milk of any total solids content between 25.7 and 31.5%, which will result in a product having any desired viscosity with a low coagulating rating after sterilization by any high-temperature short-time process.

PART II—ADEQUATE STERILIZING PROCESSES AND STABILITY IN STORAGE

The instability of the proteins in high-short sterilized evaporated milk, which shows up during storage, has already been mentioned. It is a

"setting up," or jellification, to a smooth custard-like consistency, which occurs without the agency of bacteria. The only instance known to the author of this condition having been encountered commercially, with regular methods of processing, is in connection with British standard (31.5% total solids content) evaporated milk which had been in storage at high temperature.

Processing with heat produces a progressive change in evaporated milk. The process must reach a certain point in severity to effect this change to a sufficient degree to prevent jellification or rapid cream separation during storage. The nature of the change is not known. It may be entirely physical, entirely chemical, or both physical and chemical.

The severity of the heat treatment, which is sufficient to prevent jellification of the product in storage, is lower than that which is necessary to render the evaporated milk as stable against cream separation as is the ordinary commercial evaporated milk.

The severity of the process required to prevent jellification is higher for British standard than for American standard evaporated milk.

The major part of high-short process work with evaporated milk, with which this portion of the present report is concerned, was done with the use of a process to which was applied the designation PFC—pressure filling and closing. Ball *(1)* described the process as one in which the milk was heated to the maximum temperature of the process in less than 15 seconds and immediately filled into a can and sealed. The can was violently agitated until the interior surfaces of the can and milk were sterilized. Then the can and its contents were cooled.

Information given in the PFC work and the work with the agitated can process previously described gave indications as to the degree of severity of processes that is necessary in order to preclude jellification and cream separation. These indications are shown by curves in Figure 10 for both the PFC and the agitated can methods of processing.

In Figure 10, curve C designates the various combinations of time and temperature which are known as the theoretical pressure filling and closing processes. These are the basic processes of the experimental work that has been done in the development of the pressure filling and closing method for evaporated milk. They have been shown to have the sterilizing value required to produce a commercially sterile product, with a reasonable factor of safety.

The points, L, M, and N, respectively, represent three PFC processes which were found to be necessary and sufficient to prevent the jellification of American standard evaporated milk during a storage period of 8 months at 98°F. (36.67°C.). Curve D, which is located by the points L, M, and N, therefore designates the combinations of time and temperature which constitute the processes by the PFC method that are necessary and sufficient to prevent jellification of American standard evaporated milk in storage. The corresponding curve for British standard evaporated milk would be slightly higher on the chart than Curve D. For example, evaporated milk of 32.0% total solids content, which had been processed 55 seconds at 273°F. (133.89°C.), had started to jell slightly after 8½ months of storage at

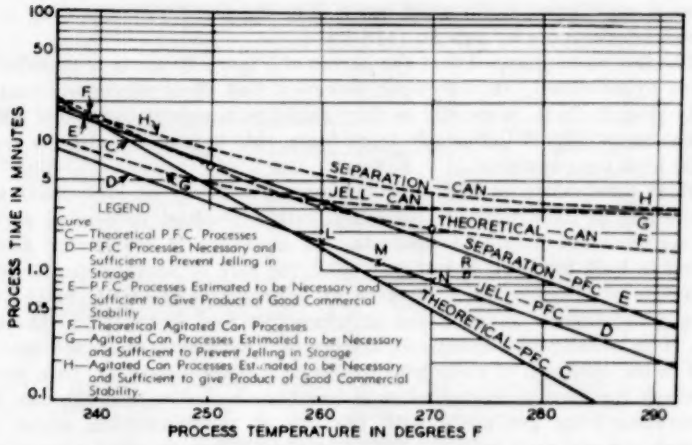

Figure 10. Curves showing minimum time and temperature of P.F.C. processes and of agitated can processes which can be used for American standard evaporated milk to give a product of commercially satisfactory stability against cream separation and jelling.

temperatures of from 75° to 98°F. (23.89° to 36.67°C.). This process is designated on Figure 10 by the point R.

Curve E is parallel to curve D and designates processes that should give evaporated milk of commercially satisfactory stability from the standpoint of cream separation. Curve E was located partially on the basis of hypothetical considerations. On the basis of commercial experience, it is assumed that the theoretical process of 13.2 minutes at 240°F. (115.6°C.) will give a commercially stable product. (Please bear in mind that this means subjecting the milk to the temperature of 240°F. for 13.2 minutes after the lapse of less than 15 seconds in bringing the milk to that temperature.) Curve E was drawn through the point representing the process of 13.2 minutes at 240°F. It was drawn parallel to curve D because the two phenomena of jelling and cream separation appear, in remarkable degree, to parallel each other in occurrence.

It is reasonable to assume that the same processes (considered from the standpoint of sterilizing value) that will be sufficient to prevent cream separation in American standard will be sufficient for the same purpose in British standard evaporated milk.

Curves F, G, and H, for evaporated milk sealed in 14-½ ounce cans before the process and kept in violent agitation during the process (as previously described), correspond respectively to curves C, D, and E which represent evaporated milk processed by the PFC method. Curve F was obtained by calculating processes at 250°, 260°, and 270°F. (121.1°, 126.7°, and 132.2°C.), equivalent to the theoretical PFC process. Curves G and H were located entirely by estimation. Curve G was drawn to intersect curve F at the point corresponding to the process temperature of 257.4°F. (125.2°C.) which is the same as the point of intersection of curves C and D.

Curve H was drawn to intersect curve F at the point corresponding to the process temperature of 240°F. (115.6°C.).

The fact is emphasized that the curves of Figure 10 are to a considerable degree hypothetical. It is thought, however, that their representations are fairly close to facts, especially as they apply to temperatures below 260°F. If the curves depart materially from facts, this is true only in the region of the high temperatures.

Heat penetration in high-short process. The study of heat penetration rate is just as important in connection with high-short processing as with the conventional long-hold methods. In processes in which the food is heated in bulk before it is put into cans, the rate of heating can be tested by means of thermocouples inserted at appropriate locations in the food heating apparatus. In processes in which the food is heated in the open can, it is usually not a difficult matter to insert thermocouples into the food in the open can to make heat penetration determinations. In a process in which the food is heated after it has been sealed within the can, heat penetration tests are made with the use of thermocouples, which pass through the can wall with the aid of stuffing boxes, in approximately the same manner as that followed in making heat penetration tests in conventional long-hold processes of any food. Because cans in this type of high-short process must be violently agitated, it is necessary to modify the "still-cook" method of making heat penetration tests by incorporating into the equipment set-up a rotary contactor for making contact with wires installed within the rotating part of the processing mechanism.

Heat penetration in agitated can process. Extensive heat penetration data on evaporated milk were obtained in the agitated can method of processing.

Heat penetration was measured in cans in the two positions, *a* and *e*, in the experimental Fort Wayne sterilizer. The entire range of rotation speed was covered. Blends that gave all variations in final viscosity and process temperatures of 240°, 243°, 250°, 260°, and 270°F. (115.6°, 117.2°, 121.1°, 126.7°, and 132.2°C.) were used. The evaporated milk studied was of two different concentrations, namely 25.7% and 31.5% total solids content, respectively.

Figure 11 shows typical heating and cooling curves plotted on special lethal rate paper constructed by Schultz and Olson *(3)*. The two curves represent two cans in the same run, one can in position *3a*, and one can in position *69e*. Milk temperature in both cans was about 50°F. (10°C.) when steam was turned on at the time indicated by the zero point of the process time scale. Steam in the retort reached 260°F. (126.7°C.) one minute after it was turned on. Rotation speed was 66 r.p.m. The ratio of the areas enclosed by the curves for the two cans gives the comparison of the two processes in sterilizing value.

$$\frac{\text{Process of can } a}{\text{Process of can } e} = \frac{4.20}{3.88}$$

The numerical values in the second member of the above equation are "F-values," used in evaluating processes to express sterilizing value. If the speed of rotation in this process had been higher, the difference between F-values of the two cans would have been greater.

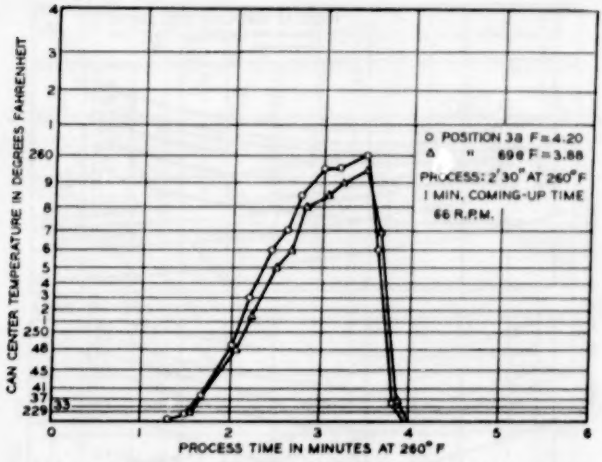
CAN CENTER TEMPERATURE IN DEGREES FAHRENHEIT
PROCESS TIME IN MINUTES AT 260° F
o POSITION 38 F=4.20
Δ " 696 F=3.88
PROCESS: 2'30" AT 260° F
1 MIN. COMING-UP TIME
66 R.P.M.

Figure 11. Heat penetration results plotted to lethal rate scale for 25.7% total solids milk in agitated can process—two identical samples in different locations within the reel.

From the data obtained from the complete set of heat penetration tests on American standard evaporated milk (25.7% total solids), F-values were calculated for processes at 270° and 260°F. The calculated values are indicated by curves in Figure 12. Each curve represents a given value of F, which is indicated on the graph. The value of 4.5 is customarily regarded as that of the minimum safe process for evaporated milk. It is the value of the theoretical processes represented by curves C and F in Figure 10. An interesting feature of this chart is the difference in trend between curves for agitation *a* and those for agitation *e*, with respect to reel speed. This characteristic is a result of the principles previously discussed with reference to the effect of position of the can. The calculations were based on thermal death time slope (z-value) of 21.

The data for cans in position *a* are presented in another form in Figure 13. These curves, like those of Figure 12, indicate conditions of uniform sterilizing value but are based on a constant speed of rotation and variable process temperature, whereas those in Figure 12 are applied to constant process temperature and variable rotation speed. In both cases, time, as representing length of process, is a variable. Figure 13 shows the following processes to have an F-value of 4.5 at the rotation speed of 66 r.p.m.: 4.7 minutes at 250°F. (121.1°C.), 2.6 minutes at 260°F. (126.7°C.), 1.2 minutes at 270°F. (132.2°C.), 0.65 minutes at 280°F. (137.8°C.), and 0.38 minutes at 290°F. (143.3°C.). These process times are exclusive of the one minute used for bringing up the temperature of the retort, that is, the F-value of 4.5 for each process includes the F-value which is contributed by the come-up time.

In the agitated can process, uniform head-space in the cans is a factor of prime importance in obtaining a uniform rate of heat penetration. In this study, the filling of every can was carefully controlled. Special head-

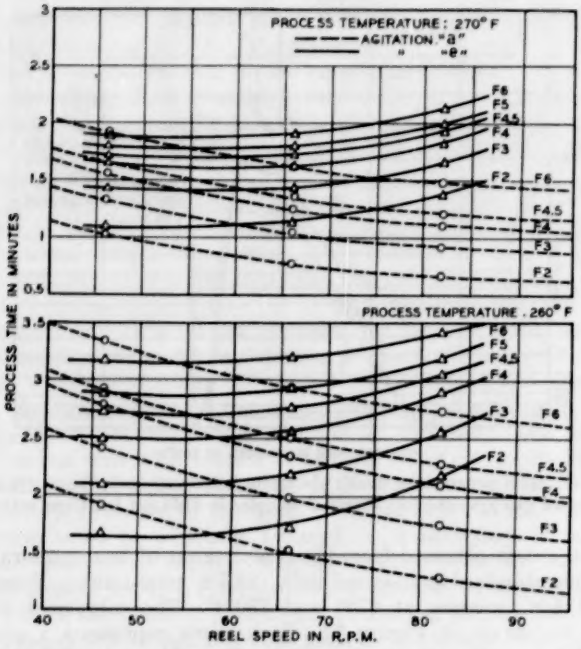

Figure 12. Effects of variations in reel speed and process time on F values for evaporated milk sterilized in the agitated can process.

space bars were used. The head-space of milk at 40°F. (4.4°C.) in the can before sealing was 31/64 inch. With milk at 75°F. (23.9°C.) after the can was processed and opened, the head-space measured 29/64 inch from the top of the double seam. Each can contained 14.5 ounces avoirdupois of milk.

When correlated with organoleptic grading of the samples, data from heat penetration tests and spoilage in inoculated packs with the agitated can process revealed conclusively that a product meeting the industry standards for sterility and equal in flavor to pasteurized milk cannot be produced by this type of process. Sterile milk of better quality than that resulting from use of this type of process can be produced by high-short processes of all other types. With the judgment of tasters naturally figuring prominently in any conclusion on this matter, indications are that processes in which either all or a major portion of the sterilization is accomplished before the milk is filled into the containers are the only ones capable of sterilizing milk in accordance with accepted standards, and at the same time retaining organoleptic qualities, when canned, comparable to those of pasteurized milk.

SUMMARY

The advantage of high-short sterilization in the preservation of high quality foods depends upon the employment of a high rate of heat pene-

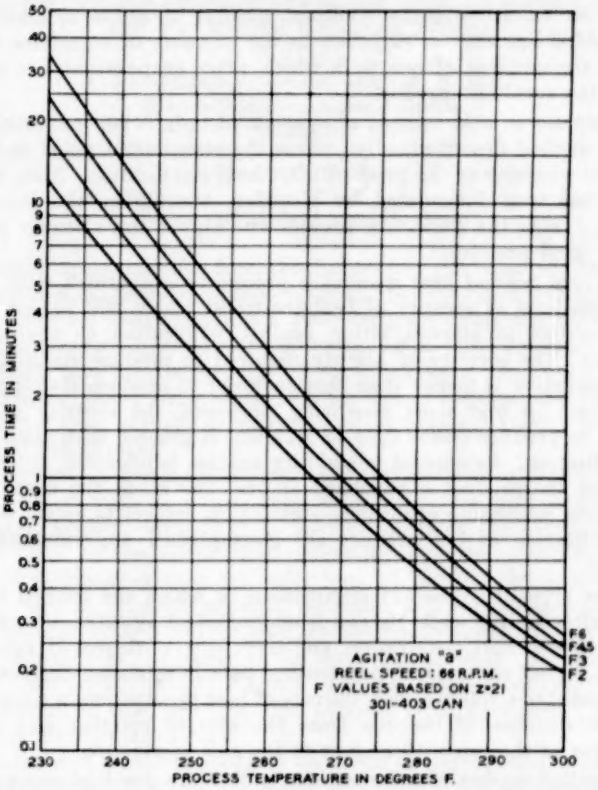

Figure 13. Average results of heat penetration tests on 25.7% total solids milk, showing equivalent processes.

tration to obtain promptly the extreme lethal effect of temperatures in the high range.

High-short sterilization of evaporated milk seems to offer greater promise of commercial success than high-short sterilization of fluid milk. Sterile evaporated milk can be produced which, when reconstituted, will meet practically every demand as a substitute for pasteurized milk, but its shelf life will be shorter than that of most other sterile foods, because of instability of texture and flavor. Texture stability can be improved by producing high viscosity in the milk during processing as well as by other measures.

High viscosity without coagulation can be produced in high-short processed evaporated milk by the addition of lactic acid to the raw milk before evaporation. In this procedure, it is advantageous to mix evaporated milk that has been acid treated with untreated evaporated milk or with evaporated milk that has been treated before evaporation with an alkaline corrective. The effects upon viscosity and coagulation produced during a heat process of evaporated milk from a given batch of raw milk depend

not only on variation in the resultant amount of acidic or alkaline corrective added but also on variation in the amounts of corrective material added to the portions of raw milk which, after evaporation, are mixed to produce the resultant product.

An increase in acid content of evaporated milk, when produced by the blending method described, often raises the coagulation point and results in reduced viscosity of the product after heat sterilization. Also, when the product has been formulated by blending, the higher the intensity of agitation during the sterilizing process, the higher the viscosity produced in the finished product.

For every type of heat sterilizing process of evaporated milk, there is a minimum level of severity of heat treatment which will impart stability to the product in storage, either against jellification or against cream separation. The severity of heating required to produce stability against cream separation is higher than that required to produce stability against jellification. In high-short sterilizing processes, the severity of heating required to produce either type of stability is greater than that required for sterilization. As applied to stability against jellification, the reverse is true when the product is sterilized at 240°-250°F. in the usual manner. Under these processing conditions, stability in respect to cream separation and sterilization of the product are accomplished approximately simultaneously.

In the type of high-short sterilization in which the food is heated in the can after sealing while the can is under violent agitation it is important that the rate of heat penetration, and therefore the degree of agitation, be identical for all cans. Heat penetration tests have shown that, when cans are processed in a rotating reel, the rate of heat penetration in a can depends upon the distance of the can from the axis of rotation and upon the orientation of the can with respect to the axis of rotation.

Controlled market distribution seems desirable for high-short sterilized evaporated milk to ensure consumption within the period of texture and flavor stability—which may be as long as six months.

Acknowledgment

The author expresses his indebtedness to R. G. Pittman, who, as a member of the staff of the Research Department of American Can Company, had charge of experimental projects on evaporated milk, to J. M. Jackson, who had charge of more recent experimental projects, to P. Sognefest, who analyzed some of the data used in this report, constructed the graphs of Figures 1, 11, 12, and 13 and offered valuable suggestions, and to F. F. Fitzgerald, now deceased, and H. A. Benjamin for valuable suggestions.

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THE STATE OF COMBINATION OF THE CAROTENOIDS OF VALENCIA ORANGE JUICE

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An earlier communication from this Laboratory *(1)* reported that saponified carotenoids of Valencia orange juice can be separated into 6 fractions by countercurrent distribution in a 200-tube glass Craig apparatus, using two different solvent systems. Chromatography of these fractions resulted in separation into 25 components, 18 of which were apparently distinct carotenoids, the other 7 stereoisomers *(1, 2)*. Ten of these had not previously been reported from oranges or orange juice; 4 were apparently previously undescribed carotenoids.

Xanthophylls in fruits differ from leaf xanthophylls in that they occur mainly as esters such as physalien and helenien (zeaxanthin and lutein dipalmitates, respectively) *(3)*. In almost all cases the identity of the combined acids in the xanthophyll esters is unknown. Zechmeister and Cholnoky *(8)* reported that the color wax of paprika on saponification yielded a mixture of oleic, myristic, palmitic, and carnaubic acids. It is probable that the acids combined with the xanthophylls are mixtures similar to those occurring in the lipids of the same fruit. Swift *(6)* investigated the lipids from freshly canned Florida Valencia orange juice and found, in order of decreasing abundance, linoleic, palmitic, oleic, palmitoleic, linolenic, stearic, and myristic acids.

Natarajan and Mackinney *(4)* on the basis of partitioning experiments stated that about 66% of the pigments in Valencia orange juice occurred as xanthophyll esters, 25% as free xanthophylls, and 9% as non-saponifiable epiphasic pigments. In earlier work at this laboratory *(1)*, it was shown that on countercurrent distribution of saponified orange juice carotenoids with the system petroleum ether-methanol (absolute or 99%) separation into 3 fractions occurred. These fractions were shown to consist of compounds containing zero, one, and two or more free hydroxyl groups, respectively. The unsaponified orange juice carotenoids were likewise separated into 3 fractions with distribution coefficients similar to those of the corresponding fractions obtained from the saponified carotenoids; it is probable that these fractions also consisted of compounds containing zero, one, and two or more free hydroxyl groups, respectively. In the present investigation the composition of these 3 fractions of the unsaponified carotenoids was studied in order to see in which fraction or fractions the various carotenoids occurred; from this it was possible to deduce whether a given xanthophyll occurs free, or as a monoester, diester, etc. It was found that xanthophylls generally occurred both as free alcohols and as esters, principally the latter; xanthophyll epoxides were found to be present mainly as esters, whereas the isomeric furanoxides occurred free to a much greater extent.

EXPERIMENTAL RESULTS

Countercurrent distribution apparatus, solvent system, and procedures for countercurrent distribution and chromatography used in the present work were similar to those described previously *(1)*. California Valencia oranges obtained in September, 1950, were hand-reamed, the juice was screened, deaerated, canned *in vacuo*, and stored at –23°C. This juice was considerably higher in carotenoids (41 mg. per liter, as β-carotene) than juice used in earlier work *(1, 2)*. Juice was thawed when needed, filtered with filter aid, the filter cake extracted with acetone, the extract evaporated *in vacuo* and the residue extracted in turn with petroleum ether and ethyl ether.

Countercurrent distribution of unsaponified carotenoids from orange juice. Two duplicate 99-transfer runs were carried out in the Craig countercurrent distribution apparatus, using the system petroleum ether-99%

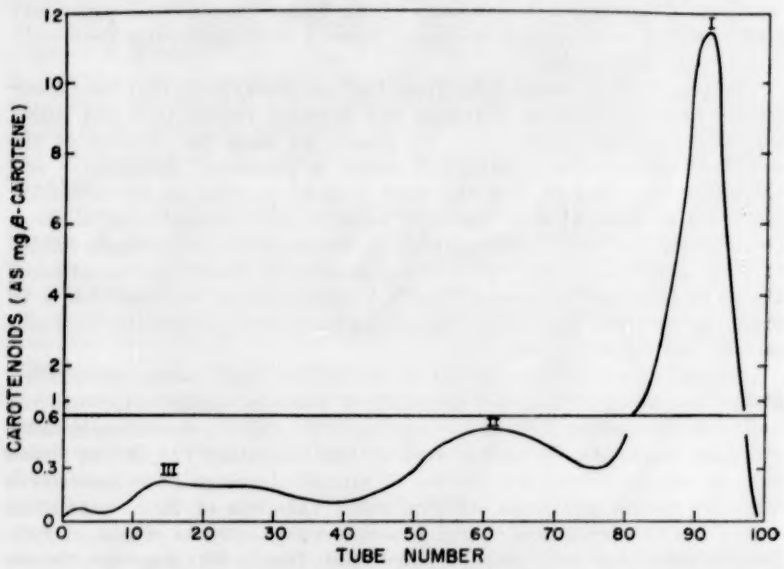

Figure 1. Countercurrent distribution of unsaponified orange juice carotenoids with the system petroleum ether-99% methanol.

methanol (1.8 to 1 by volume). The two layers were equilibrated, and 10 ml. of each layer used in each tube of the apparatus. In the first run, the color was measured in each of the 100 tubes of the Craig apparatus (after adding acetone to 25 ml. to make homogeneous) in an Evelyn photoelectric colorimeter using the 440 mμ filter, and was calculated as β-carotene *(1)*. The distribution obtained is illustrated in Figure 1 and showed a separation into 3 fractions: I, no free hydroxyl group; II, one free hydroxyl; and III, two or more free hydroxyls. The percentages of the total carotenoids in these 3 fractions (as β-carotene) were: I, 82%; II, 12%; and III, 6%.

The 100 solutions were then combined into 3 fractions: tubes 0-38 (III), 39-75 (II) and 76-99 (I). In the first run the unsaponified fractions were chromatographed directly, while in the second run the 3 fractions were saponified before chromatographing.

Chromatography of unsaponified fractions. Each of the 3 fractions was evaporated *in vacuo*, dissolved in petroleum ether (I and II) or benzene (III), and chromatographed on magnesia (Westvaco No. 2642) plus diatomaceous earth (1 + 3 by volume).

Fraction I. (No free hydroxyl). The column was eluted successively with 1½, 3, 5 and 10% acetone in petroleum ether, and the residual pigments then removed with 5% ethanol in petroleum ether. Ten fractions were collected, the first being the colorless eluate preceding the first colored band; this fraction contained a much larger amount of viscous, non-volatile material than in the corresponding fraction from saponified lipides. It was suspected that this colorless material was probably chiefly triglycerides, although these have not been shown to be present in orange juice lipides *(7)*. Fractions 3, 6, and 8 were red bands on the column (probably mainly β-carotene, cryptoxanthin esters, and zeaxanthin diesters, respectively), fractions 2, 5, 7, 9 and 10 were orange, while 4 was yellow. The absorption spectra of fractions 1, 2, 3, and 4 corresponded closely to those of phytofluene, α-, β-, and zeta-carotenes, respectively, which had previously been found in orange juice *(1, 4)*. These bands were cleanly separated from the other bands, which contained xanthophyll esters. Fractions 5 to 9 appeared as distinct bands during the elution, but they did not separate cleanly even on rechromatographing. On saponification and rechromatography, all proved to be mixtures. The xanthophyll ester mixture was much more difficult to separate than the saponified xanthophylls, probably due to the presence of much more colorless material and to the considerably greater number of colored components; it is very probable that there were several different esters of each xanthophyll.

Fraction II. (One free hydroxyl). Chromatography of this fraction was considerably more satisfactory than that of fraction I; a number of rather pure sub-fractions were obtained, some of which proved to be partially esterified xanthophylls. The second band eluted gave a spectral absorption curve indicating it to be a rather pure substance with absorption maxima in petroleum ether at 439, 414, 396, and 306 mμ. It was apparently an unknown carotenoid. On recombination of the entire fraction II, saponification and rechromatography, this sub-fraction was not located, nor has a simliar fraction ever been isolated by the present investigators from saponified orange juice lipides. Perhaps it was an ester of a carotenoid acid (see below).

Fraction III. (Two or more free hydroxyls). Chromatography of this fraction was quite unsatisfactory, the only reasonably pure sub-fraction obtained being auroxanthin. Considerable colorless material occurred in this fraction, probably mainly phospholipides which were found to be a major part of the lipide fraction of orange juice *(7)*.

Chromatography of fractions after saponification. Contents of the 100 tubes of the second countercurrent distribution run were combined into 3 fractions as before, which were evaporated in vacuo, saponified, dissolved in benzene, and chromatographed on columns of magnesia plus diatomaceous

earth (1 + 1 by volume). The carotenoids obtained together with their approximate percentages are given in Table 1. Identifications were based on knowledge of compounds previously found in orange juice *(1, 2)*, behavior on chromatography, spectral absorption curves, and on hydrochloric acid-ether tests. In several cases a given component was obtained in two or more stereoisomeric forms as in earlier work *(2)*; these are combined in Table 1, as with "mutatoxanthins." Spectral absorption maxima are omitted from Table 1 since in most cases they were essentially the same as those previously published *(1, 2)*. The relative amounts of the various components were calculated from the optical density of the spectral absorption curves at the principal maxima, and from the volumes of the original solutions and the dilutions, if any, which had been made when the absorption curve was run. Due to the complexity of the xanthophyll fraction *(2)*, some of the fractions obtained were impure and it is probable that some of the constituents present were not isolated. For example, in previous work lutein, zeaxanthin, antheraxanthin, cis-antheraxanthin, violaxanthin, and cis-violaxanthin were found in saponified orange juice lipides, but we were

TABLE 1

Carotenoids obtained from unsaponified Valencia orange juice lipids (on countercurrent distribution, combining into 3 fractions, saponification and chromatography)

No.	Probable identity[1]	Approximate percentages in			
		Fraction I (no free OH)	Fraction II (one free OH)	Fraction III (> one free OH)	Total
1	Phytoene	0.6	....	....	0.6
2	Phytofluene	0.5	....	....	0.5
3	α-Carotene	0.6	....	....	0.6
4	β-Carotene	1.3	....	....	1.3
5	zeta-Carotene	3.1	....	....	3.1
6	OH-α Carotene-like	2.8	0.5	....	3.3
7	Cryptoxanthin	3.3	1.5	....	4.8
8	Lutein	5.8	1.1	0.5	7.4
9	Zeaxanthin	4.8[2]	?	0.8	5.6
10	Antheraxanthin	3.8[2]	4.4[2]	....	8.2
11	Mutatoxanthins	0.6[2]	0.2	0.6	1.4
12	Violaxanthin	34.9[2]	?	....	34.9
13	Luteoxanthins	5.9	9.3	0.1[3]	15.3
14	Auroxanthin	....	....	2.1	2.1
15	Sinensinxanthin	1.7	0.3	....	2.0
16	Trollein	....	....	1.3	1.3
17	Trollixanthin-like	0.1[2]	1.7	2.2	4.0
18	Valenciachrome	0.3	....	....	0.3
19	Trollichrome-like	....	0.5	0.8	1.3
	Unidentified	0.1	1.9	....	2.0
	Total	70.2	21.4	8.4	100.0

[1] Based on behavior on chromatography, special absorption curves, and on hydrochloric acid-ether tests.

[2] Fractions not cleanly separated, values may be considerably in error.

[3] Later work indicates this may be flavoxanthin.

Nos. 1-5 are hydrocarbons; 6-7, monols; 8-9, diols; 10-11, moncether diols; 12-14, diether diols; 15-19, polyols.

unable to separate this mixture chromatographically at all satisfactorily when they were all present in a single solution, as probably was the case in Fraction I and perhaps also in Fraction II. The value of violaxanthin in Fraction I is probably somewhat high, and the values for zeaxanthin and antheraxanthin somewhat low, judging by results obtained with other samples.

The hydrocarbons phytoene, phytofluene, α-, β-, and zeta-carotenes were found in Fraction I only. The eluate which came out ahead of the phytofluene band (which had a brilliant green fluorescence in ultraviolet light) had a bluish fluorescence; the spectral absorption curve in ethanol had maxima at 284 and 273 mμ, with a hump at 300 mμ, resembling closely the curve for phytoene which was found in oranges by Rabourn and Quackenbush *(5)*.

Cryptoxanthin and the hydroxy-α-carotene-like substance were found in Fraction I and to a lesser extent in Fraction II, indicating that they occurred mainly as esters. Similarly, lutein and zeaxanthin were found chiefly as diesters; some lutein was found as a monoester in Fraction II, and both occurred as free xanthophylls in Fraction III.

The monoepoxide of zeaxanthin, antheraxanthin, was found in Fractions I and II, while the isomeric furanoxide, mutatoxanthin, was found in all three. Violaxanthin, the diepoxide of zeaxanthin, was found only in Fraction I, while the isomeric epoxide-furanoxide luteoxanthins were apparently found in all three, but mainly in II (more recent work indicated that the luteoxanthin-like substance in Fraction III may have been flavoxanthin); the isomeric difuranoxide auroxanthin was found only in Fraction III. Xanthophyll epoxides are converted to the isomeric furanoxides in the presence of acids *(3)*; it is probable that only the epoxides are synthesized by enzymatic action in the plant. The results obtained in the present investigation indicate that the free xanthophyll epoxides are more readily isomerized to the furanoxide than are the xanthophyll epoxide esters.

The epoxide-furanoxide polyol pair tentatively identified as trollixanthin and trollichrome were found almost entirely in Fractions II and III, indicating that these substances did not occur in a fully esterified form. The nonether polyol, for which the name *trollein* was proposed *(2)*, was found in the present work in Fraction III, whereas it was previously obtained as a product of the reaction of hydrochloric acid on the trollixanthin-like fraction. The spectral absorption curve of this substance is similar to those of lutein and antheraxanthin, but from its behavior on countercurrent distribution and chromatography must contain more than two hydroxyl groups. Possibly the structural formula is like that of antheraxanthin with the epoxide group replaced by two hydroxyl groups.

The substance for which the name valenciachrome *(2)* was proposed was found only in Fraction I, indicating it to be fully esterified, though it is apparently a polyhydroxy furanoxide. The corresponding epoxide valenciaxanthin *(2)* was not found in the present work, probably being overlooked because of its faint color.

Acidic pigments. On saponification of orange juice lipides by potassium hydroxide in methanol-ethyl ether, followed by dilution with water and repeated extraction with ethyl ether, a significant amount of color usually

remained in the aqueous layer. After acidification the color was extractible with ether, but on extracting the ether layer with alkali, the color returned to the water layer. This indicated the presence of an acidic pigment in the lipide fraction after saponification. On adsorption from benzene onto a column of aluminum oxide, the color was not eluted even by absolute alcohol; on elution with 0.1% formic acid in benzene, 3 fractions were obtained. The spectral absorption curves of these fractions indicated that they were mixtures; the last two eluted had maxima in benzene at *ca* 440, 410, and 386, and *ca* 435, 403, and 380 $m\mu$, respectively, indicating the presence of compounds containing about seven conjugated double bonds. No such fractions were obtained on chromatographing the unsaponified lipides, suggesting the initial occurrence as an ester. The above confirms the observation of Swift *(6)*, who reported a red acidic pigment in the unsaponifiable fraction of Florida Valencia orange juice.

SUMMARY

The composition of the three major carotenoid fractions separated on countercurrent distribution of unsaponified Valencia orange juice lipides with the system petroleum ether-99% methanol has been investigated. The major part of the xanthophyll fraction was found to occur in a fully esterified form. The xanthophylls not containing cyclic ether groups (cryptoxanthin, the hydroxy-α-carotene-like fraction, lutein, zeaxanthin) occurred mainly as esters. Xanthophyll epoxides occurred principally as esters, the isomeric furanoxides to a considerably greater extent as free alcohols.

Two carotenoids not previously found in orange juice or apparently elsewhere in nature were discovered. One was a polyol for which the name *trollein* was proposed. The other was found on chromatographing unsaponified lipide material and may have been an ester of a carotenoid acid. The presence of acidic pigments after saponification was confirmed.

Acknowledgment

The assistance of Stanley Friedlander in running the spectrophotometer curves is acknowledged.

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